## COMMENTARY

# Leaping Forward in Amphibian Health and Nutrition

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The Epidemiology Working Group, a subgroup of the participants of the Disney's Animal Kingdom Workshop on "Ex situ Amphibian Medicine and Nutrition," identified a critical need to design and implement approaches that will facilitate the assessment and evaluation of factors impacting amphibian health. In this manuscript, we describe and summarize the outcomes of this workshop with regards (a) the identified gaps in knowledge, (b) identified priorities for closing these gaps, and (c) compile a list of actions to address these priorities. Four general areas of improvement were identified in relation to how measurements are currently being taken to evaluate ex situ amphibian health: nutrition, infectious diseases, husbandry, and integrated biology including genetics and endocrinology. The proposed actions that will be taken in order to address the identified gaps include: (1) identify and quantify major health issues affecting ex situ amphibian populations, (2) identify and coordinate laboratories to conduct analyses using standardized and validated protocols to measure nutritional, infectious diseases, genetic, and hormonal parameters, (3) determine in situ baseline distribution of parameters related to amphibian health, and (4) establish an inter-disciplinary research approach to target specific hypotheses related to amphibian health such as the effects of population genetics (e.g., relatedness, inbreeding) on disease susceptibility, or how environmental parameters are related to chronic stress and hormone production. We think is important to address current gaps in knowledge regarding amphibian health in order to increase the probability to succeed in addressing the issues faced by in situ and ex situ amphibians populations. We are confident that the recommendations provided in this manuscript will facilitate to address these challenges and could have a positive impact in both the health of in situ and ex situ amphibian populations, worldwide. Zoo Biol. XX:XX–XX, 2014. 

**Keywords: amphibian health; husbandry; nutrition; pathology; research; vitamin A; water; zoos; aquariums; ex situ populations**

## INTRODUCTION

Prior efforts conducted by colleagues in different fields (zoo keepers, researchers, academicians, veterinarians, nutritionists, biologist, geneticists, conservation groups, to mention some) and by different institutions worldwide have resulted in different approaches without having well-defined standards, techniques, tools, measurements units, and protocols in place to evaluate different aspects related to amphibian health [Ferrie et al., 2014]. This lack of standardization represents a significant challenge when evaluating parameters associated with the health and nutritional status of different amphibian species. The objectives of this

Recommendations from the Veterinary Medicine, Husbandry, Epidemiology, Nutrition and Science and Research, Working Groups of the Ex Situ Amphibian Medicine and Nutrition Workshop (February 2013).

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Received 05 February 2014; Revised 11 August 2014; Accepted 09 September 2014

DOI: 10.1002/zoo.21178
Published online XX Month Year in Wiley Online Library (wileyonlinelibrary.com).

manuscript are to describe and summarize the outcomes of the Disney's Animal Kingdom Workshop on "Ex situ Amphibian Medicine and Nutrition" [Valdes et al., 2014] with regards to (a) the identified gaps in knowledge, (b) identified priorities for closing these gaps, and (c) compile a list of actions to address these priorities. We provide recommendations based on the outcomes of the work conducted at this workshop and the literature overview conducted by Ferrie et al. [2014] and included as the first manuscript in this Zoo Biology special edition.

We think there is a critical need to conduct research and implement approaches (i.e., sampling techniques) and methodologies (i.e., laboratory methods) that will facilitate the evaluation of factors impacting amphibian health. This work needs to be designed and implemented by trained personnel including expertise from different disciplines in collaborative approaches (i.e., nutritionist, laboratory technicians, veterinarians) in order to gain comprehensive knowledge in different areas. Finally, differences among amphibian species and various life stages should be considered when conducting this research.

Four general areas in which improvement is possible with regards to amphibian health were identified based on current ex situ amphibian health evaluation practices: (1) nutrition, including nutritional diseases, (2) infectious diseases, (3) husbandry, and (4) integrated biology focusing on genetics and stress hormones. To address this challenge, four priorities were identified to understand different factors impacting amphibian health [Ferrie et al., 2014]: (1) identify and quantify major health issues affecting ex situ amphibian populations, (2) identify and coordinate laboratories to conduct analyses using standardized and validated protocols to measure nutritional, infectious diseases, genetic, and endocrinology parameters, (3) determine in situ (in the wild in their natural environments) baseline distribution of parameters related to amphibian health, and (4) establish an interdisciplinary research approach to target specific hypotheses related to amphibian health such as the effects of population genetics (e.g., relatedness, inbreeding) on disease susceptibility, or how environmental parameters are related to chronic stress and hormone production.

The outcomes of this workshop indicate that serious obstacles still prevent us from understanding the factors affecting amphibian health. Furthermore, some of the same challenges and concerns expressed 8 years ago [Zippel et al., 2006] still remain as important constraints. The identified gaps in knowledge and list of actions for closing these gaps identified at this workshop are summarized and described in the next four sections.

## RECOMMENDATIONS

### Identify and Quantify Major Health Issues Affecting Ex Situ Amphibian Populations

To date, there are knowledge gaps in identifying the main health problems affecting ex situ amphibian populations worldwide [Ferrie et al., 2014]. To address this challenge, we recommend conducting a retrospective review of health and husbandry records from facilities holding amphibians. The members of the Epidemiology Working Group have developed a survey which will acquire information regarding the four major areas impacting amphibian health identified at the workshop: nutrition, infectious diseases, husbandry, and integrated biology including genetics and endocrinology. With the collaboration of the Amphibian Ark, this survey can be administered to all known facilities holding amphibian collections worldwide. Priority will be placed in obtaining health baseline parameters in different regions. We recommend comparing parameters related to amphibian health between developed and developing areas of the world, and obtaining information for different amphibian species and life stages. In addition, emphasis should be placed on collecting husbandry information regarding microhabitat utilization, seasonal changes and environmental parameters in order to assess captive care techniques and their potential impact on health. Technologies used for lighting (including UV-B radiation), water quality, feeding approaches, and population management programs should also be evaluated.

We suggest critically evaluating and assessing the content of the obtained data by determining the quantity and quality of information available regarding amphibian health. In addition, identifying facilities lacking the ability to obtain and store information regarding amphibian health properly could be quantified. At the completion of this survey, it is our expectation that we would be able to determine the frequency of different parameters related to amphibian health, thus enabling us to identify specific health problems in each of the four main areas identified as impacting amphibian health. We suggest the implementation of this survey as a critical first step to identify and quantify the major health issues affecting ex situ amphibians collections. Complementing this survey, a comprehensive literature review needs to be conducted for each of these four areas under investigation. This will allow prioritizing and directing subsequent endeavors and streamlining efforts thus minimizing the financial impact.

### Identify and Coordinate Laboratories to Conduct Analyses Using Standardized and Validated Protocols to Measure Nutritional, Infectious Diseases, Genetic and Endocrinology Parameters

The lack of standardization of procedures, approaches, protocols, measures, tests, and tools used worldwide was identified as a major challenge to understanding amphibian health [Clugston and Blaner, 2014; Ferrie et al., 2014]. This lack of consistency generates significant challenges to objectively and comprehensively assess (and compare) information from different sources. Thus, it is imperative that standardized and validated methods and approaches are identified, agreed upon, and utilized. The process by which methods and approaches are standardized and validated

should be specific to the method and discipline to which that method applies, and the challenge of standardization may be best tasked to the associated Association of Zoo & Aquariums (AZA) committee (i.e., AZA Animal Health Committee, AZA Nutrition Advisory Group), the European Association of Zoos and Aquaria, Latin American Association of Zoos and Aquariums among others. The effort required to accomplish this task should not be underestimated. Only then can measures of exposure factors (including husbandry and nutrition), physiological parameters, and disease status (including pathological findings) be accurately quantified to assess the health of ex situ individuals and populations. Standardized protocols also will facilitate comparisons among regions and different institutions and the creation of a centralized database, which can be accessed by researchers worldwide for the assessment of various parameters related to amphibian health.

Significant gaps also were identified regarding amphibian nutrition [Clugston and Blaner, 2014; Ferrie et al., 2014], which provide opportunities for future research efforts and potential diet and supplement development. For example, different laboratories currently are testing liver, blood, and/or whole body samples to obtain vitamin A concentrations, which are not comparable. Specifically for vitamin A analysis, we recommend the development of standard sampling techniques and assays for analysis of vitamin A/retinol/retinyl esters/carotenoids levels in serum and tissue (individual tissues and whole body for small animals). These protocols need to include considerations for sampling techniques for use in clinical scenarios as well as research settings, bearing in mind both the logistics and costs associated with each procedure [Clugston and Blaner, 2014].

Without established amphibian nutrient requirements, the determination of appropriate supplementation practices for the variety of managed species is challenging. Further complicating matters are the highly variable habitat and natural history requirements. This makes the establishment of a single nutrient profile unlikely to fit the nutritional needs of all amphibians. Although nutrition experts identified and suggested a combination of other vertebrate species that have known nutrient requirements that might provide reference to formulate current diets [Ferrie et al., 2014], additional research is necessary to make more targeted recommendations.

We suggest evaluating current dietary supplementation practices and associations with health problems in captive populations. Additionally, trials of dietary supplements are needed to evaluate the efficacy of various approaches to improve captive diets. These trials should involve multiple species to avoid "one size fits all" conclusions. For instance, some species may be easily able to utilize a wide range of different carotenoids to meet vitamin A requirements, whereas others may require only preformed vitamin A/retinol. The development of a "Prey Supplementation Best Practices Manual" is needed. While data exist on whole prey nutrients and institutional supplementation practices [Livingston et al., 2014], it was noted many existing data may not be published. The planned survey and literature review (suggested in point 1) may be useful to reach unpublished, yet useful data regarding supplementation practices currently used. Evaluation of commercial insect production systems should be included in order to assist in the development of guidelines specific to breeding and rearing insects. This assessment is needed in order to develop more nutrient-dense insects on the procurement side of nutrition management. With regards to food security, the presence of antibiotics, toxins, and infectious agents in food are important topics to be considered by insect producers, feed manufacturers, and facilities managing insects prior to their use as prey items [Ferrie et al., 2014]. Other gaps in nutrition research include the nutrient composition of water, nutrient absorption and metabolism, and the influence of nutrients on gene expression and molecular biomarkers [Ferrie et al., 2014].

## Proposed Standardized Approaches and Methods

Necropsy examination of animals that die can detect a wide range of infectious and non-infectious disease problems encountered in ex situ programs [Pessier and Mendelson, 2010; Pessier et al., 2014]. Because of the difficulty in obtaining and interpreting amphibian clinical samples, necropsy findings and diagnostic tissue samples are invaluable for health assessment of amphibian populations. Therefore, whenever possible, necropsies which include histopathology are advised for a significant proportion of deaths in ex situ programs.

When collecting necropsy tissues for nutritional, immunologic, hormonal or genetic studies animals that are freshly or recently dead are preferable and specimens with gross evidence of decomposition (e.g., discoloration, smell, discharge of fluids, bloating) should be avoided. The ideal sample is from a euthanized animal with immediate sample collection or immediate refrigeration of the carcass with sample collection occurring the same day. For collections of liver (e.g., for vitamin A analysis) as much tissue as possible should be collected while avoiding the gallbladder. In anurans this is accomplished by sampling the lateral 2/3 of both the left and right liver lobes (gallbladder is central to these lobes). Tissue should be collected into new clean plastic vials or plastic bags (e.g., Whirl-Pak bags), and immediately protected from light by wrapping in aluminum foil and frozen. The sampled tissue should be frozen immediately in liquid $N_2$, maintained at $-80°C$ and transported on dry ice to the diagnostic laboratory as soon as possible, as some indices of nutritional status may be degraded at higher temperatures [Clugston and Blaner, 2014]. Additionally, we recommend that all ex situ programs freeze plasma from all individuals that have blood drawn for health related blood panels. Some can use frozen plasma samples, and plasma can be frozen and stored for an indeterminate length of time.

Guidelines for use of diagnostic testing for specific important infectious diseases of amphibians such as

chytridiomycosis and ranaviral disease are available [Pessier and Mendelson, 2010]. As the established assays for these pathogens are improved or as new infectious disease problems are identified new diagnostic tests and recommendations will emerge. Development of well-defined parameters (laboratory-based or clinical signs) to differentiate stages of disease (e.g., subclinically infected vs. disease) or to evaluate the effectiveness of treatment protocols (e.g., vitamin A supplementation for hypovitaminosis A) will be very important.

For institutions working with amphibian populations, we recommend collecting and maintaining as much genetic material as possible for future DNA research on genetics and population diversity, as well as understanding expression of genes in relationship to nutrition and disease. Sample collections could be in the form of toe clips, skin biopsies, specific tissues, or entire carcasses stored long term in −80°C freezers, ethanol or other DNA preservatives. Establishment of amphibian cell culture lines and preservation of gametes and embryos are also important aspects of biobanking efforts [Kouba et al., 2013]. Samples should be collected from all individuals of a species if possible.

We expect that standardization of these approaches will be conducted and agreed upon by experts in each field. Furthermore, we suggest that these efforts are sponsored and supported by institutions holding amphibians (e.g., American Association of Zoos and Aquariums; European Association of Zoos and Aquaria, Latin American Association of Zoos and Aquariums among others). We suggest that key players such as nutritionists, pathologists, clinicians, epidemiologists, research biologists, and husbandry specialists among other subject matter experts, meet via conference calls in order to identify suitable laboratories, validate approaches, and standardize protocols.

## Determine In Situ Baseline Distributions of Parameters Related to Amphibian Health and Implement Comparative Studies between Ex Situ and In Situ Populations

Once the aforementioned two priorities have been met, prospective studies should be designed and implemented based on the collective information gained from recommendations 1 and 2 to ascertain baseline distributions of parameters in amphibians in situ. Considering the logistics and associated costs, different study designs could be considered and implemented [Dohoo et al., 2003] to evaluate and determine various parameters distributions in order to obtain baseline values among wild populations. We recommend two specific strategies to facilitate the work needed with in situ populations:

1. Partnerships with in situ biologists studying amphibian populations should be established and more resources and efforts should be prioritized to develop research aimed at understanding amphibian species in their natural environment.
2. Of those species with wild populations remaining, or those with active reintroduction projects, comparative studies between the in situ and ex situ populations, focusing on basic ecological questions, should be developed.

When conducting these in situ studies, knowledge obtained can be extremely beneficial when designing facilities, enclosures, feeding regimes, or reproduction systems for ex situ amphibian populations. Emphasis on obtaining information to establish the unique requirements of individual species should be considered. In addition, the impact of different environments (arboreal, terrestrial, aquatic) and seasons should be evaluated. The following are topics and areas we suggest in situ studies focus on while considering different life stages, as many of these topics and areas will be different for larvae, recently metamorphosed individuals, and adults:

–Diet: obtaining data for both the amphibians and their dietary items including prey species (determined by fecal collection, gastric washes, field observations, museum specimens, and feeding strategies).
–Water: quality parameters for quality (e.g., ammonia), composition (e.g., solute concentrations and pH) and toxicology.
–Environmental factors: such as temperature (e.g., optimal low and high temperatures), UVB light, humidity, seasonal activity (e.g., estivation, hibernation).
–Behavior and social structure: microhabitats utilized on a seasonal and/or daily basis.
–Obtaining body mass measurements from healthy, wild specimens would be helpful in monitor fitness.
–Reproduction strategies, and Diseases: affecting in situ populations.

Collecting and using information obtained from in situ populations can be extremely useful to understanding and managing factors impacting amphibian health. This information is needed in order to develop and test hypotheses aimed at decreasing the gap between ex situ and in situ populations in order to improve ex situ amphibian health and thus facilitate the establishment of survival assurance populations. This is essential in increasing the probability of implementing successful and sustainable amphibian reintroduction programs. We recommend that all information obtained from in situ studies should be considered in order to aid and guide the implementation of husbandry manuals for each species ex situ. Baseline parameters should be established within 2 years. The outcomes and information generated from these prospective in situ studies should would be used to revisit the husbandry practices related to the basics of amphibian captive care, as well for the design of future research aiming to evaluate ex situ amphibian health.

### Establish an Inter-Disciplinary Research Approach to Target Specific Hypotheses Related to Amphibian Health such as the Effects of Population Genetics (e.g., Relatedness, Inbreeding) on Disease Susceptibility, or How Environmental Parameters are Related to Chronic Stress and Hormone Production

When planning, designing, and conducting new research projects, we consider it critical to use standardized and validated laboratory methods when collecting and analyzing high quality samples (as suggested in point 2). It would be beneficial to develop a partnership with academic institutions or identify student projects that could focus on a specific research question (e.g., relatedness, inbreeding, stress related topics). General research questions should focus first on individual variability, sex differences, circadian patterns, and species differences. Once baseline information is obtained, glucocorticoid levels can be studied in conjunction with effects of environmental changes, immunological questions, prevalence of disease among others [Narayan, 2013].

We recommend investigating whether increased inbreeding, high mean kinship, and/or low genetic diversity are making some species more susceptible to some of the observed nutrition and health issues. In order to include population genetics research into ex situ management of amphibians to establish the basis for genetically healthy populations, we offer three specific recommendations in increasing scope, effort, and cost:

1. All specialists working with species should develop pertinent research questions related to understanding the genetic history and composition of their population, and include molecular genetic studies prior to or at the founding of the population. Population genetics research projects should be conducted by a population biologist/geneticist who is familiar with captive management and analysis techniques, in strong partnership with other professional disciplines. During project development, researchers should determine which markers will be useful in answering specific questions (i.e., neutral markers to study genetic diversity, relationships, and population demographic history [Beebee, 2005], or major histocompatibility complex [MHC]) to look at predictors of survival against infection [Barribeau et al., 2008; Savage and Zamudio, 2011] and how the resulting data will be analyzed and applied to answer questions.
2. Programs or institutions should support and participate in molecular genetic studies by providing funding and/or biological samples to whole genome sequencing projects for eventual gene targeted studies [Storfer et al., 2009; Hellsten et al., 2010]. Future studies should focus on comparisons of gene expression in multiple amphibian species, identification of fitness related traits [Beebee, 2005], genomic screen of amphibian gut microbes, and identification of commercially important genes, such as toxin producing genes in *Dendrobates* or antifungal or antimicrobial genes [Rollins-Smith, 2009].

With regards to amphibian ecology, research groups seeking to integrate endocrinology questions into their research (in order to assess stress) should also consider partnering with established endocrine and biochemistry laboratories equipped with gas chromatography [GC; Krone et al., 2010] or high performance liquid chromatography (HPLC) systems in order to investigate thoroughly the steroid metabolism profiles for their study species [Narayan, 2013]. Additionally, to assess stress in amphibians, we recommend evaluating endocrinology parameters in situ by initiating non-invasive endocrine studies using minimally invasive sample collection such as urine, fecal, saliva, or aquatic media samples [see Narayan, 2013]. We also recommend utilizing or testing immunoassays with antisera that cross-react with the target reproductive or glucocorticoid steroid and its primary metabolites where possible. All new species being studied should have the proper biological (physiological) and chemical assay validations performed to account for potential individual and species-specific differences in steroid metabolism and methodological considerations such as sample collection, processing and storage [Buchanan and Goldsmith, 2004; Narayan et al., 2010; Sherriff et al., 2011; Goymann, 2012; Narayan, 2013].

## CONCLUSION

As part of this multi-disciplinary and multi-institutional effort, we have identified key areas that need to be explored further in order to obtain a more complete and comprehensive understanding of the major challenges faced by amphibian populations and the factors impacting amphibian health. We think it is important to address the challenges and gaps in knowledge regarding amphibian health to increase the probability in successfully dealing with the issues faced by in situ and ex situ amphibian populations [Valdes et al., 2014; Ferrie et al., 2014]. We are confident that the implementation of these recommendations by this multi-disciplinary group can have a significant positive impact in the health of in situ and ex situ amphibian populations, worldwide.

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