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COMMENTARY

Nutrition and Health in Amphibian Husbandry

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Amphibian biology is intricate, and there are many inter-related factors that need to be understood before establishing successful Conservation Breeding Programs (CBPs). Nutritional needs of amphibians are highly integrated with disease and their husbandry needs, and the diversity of developmental stages, natural habitats, and feeding strategies result in many different recommendations for proper care and feeding. This review identifies several areas where there is substantial room for improvement in maintaining healthy ex situ amphibian populations specifically in the areas of obtaining and utilizing natural history data for both amphibians and their dietary items, achieving more appropriate environmental parameters, understanding stress and hormone production, and promoting better physical and population health. Using a scientific or research framework to answer questions about disease, nutrition, husbandry, genetics, and endocrinology of ex situ amphibians will improve specialists' understanding of the needs of these species. In general, there is a lack of baseline data and comparative information

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for most basic aspects of amphibian biology as well as standardized laboratory approaches. Instituting a formalized research approach in multiple scientific disciplines will be beneficial not only to the management of current ex situ populations, but also in moving forward with future conservation and reintroduction projects. This overview of gaps in knowledge concerning ex situ amphibian care should serve as a foundation for much needed future research in these areas. Zoo Biol. XX:XX–XX, 2014.

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INTRODUCTION

Understanding the many facets of amphibian biology is paramount in establishing a successful Conservation Breeding Program (CBP), which are mandated by the IUCN's Amphibian Conservation Action Plan for amphibian conservation and recovery plans [Gascon et al., 2007]. The Class *Amphibia* itself is a complex and diverse group, characterized by species living in most ecosystems that require some form of water at early life stages. Some of the complex issues that are not fully understood in amphibian management include the cutaneous physiology related to skin and the transport of water and nutrients via skin or gastrointestinal tract, the complex life histories with the timing of metamorphosis, and varying needs for species at each life stage. In addition to these life histories, each species also has diverse ecological needs and is sensitive to small shifts in environmental variables that can have great effects on individual or population health and persistence. The manuscript that follows is a review of the complex needs of amphibians maintained in CBPs and the current problems facing the experts who manage these populations.

Issues in Nutrition

Nutritional needs of amphibians are highly integrated with their veterinary and husbandry needs, and the diversity of developmental stages, natural habitats, and wild-type feeding strategies will result in many different recommendations for proper care and feeding. At this time, however, information is somewhat limited and thus some generalities must be made that should be refined in the future.

Nutrient Metabolism

Energy demands of amphibians vary by species and life stage [Clayton, 2005]. Resting and active metabolic rates are generally higher for anurans compared to salamanders, and for terrestrial species compared to aquatic species [Gatten et al., 1992; Hillman et al., 2009]; both metabolic rate and rate of digestion increase with ambient temperature. Rates of energy intake of amphibians have been estimated by clinical experience [Hadfield et al., 2006], although may not be consistent either over the short term (e.g., number of meals eaten in a week) [Jorgensen, 1989], or longer time frames (e.g., pronounced annual rhythm in food intake with a period of hyperphagia, growth, and fat deposition followed by a period of aphagia and weight loss). Correspondingly, the mass of the digestive system and associated energy requirements may change several fold during the annual cycle [Naya and Bozinovic, 2004], such as during aestivation or hibernation, for entrainment of reproduction [Brenner and Brenner, 1969; Browne and Zippel, 2007]. Energy deposition in the form of fat (body condition), along with hibernation periods, have been directly linked to reproductive output including fecundity and fertilization success [e.g., Brenner, 1969; Browne et al., 2006; Browne and Zippel, 2007] and thus should be carefully considered in the nutrients fed ex situ amphibians.

Water balance is critical to amphibian nutrition. In general, amphibian skin is highly glandular and vascularized but has a reduced stratum corneum, which makes it permeable to water and many fat-soluble compounds. The skin is also a major site for electrolyte uptake or loss depending on the osmolarity of the water or soil in the habitat [Cereijido et al., 1964; Farquhar and Palade, 1964; Campbell et al., 2012]. Water salinity can represent a major physiological stress in amphibians [Bennett and Johson, 1973; Balinsky, 1981; Boutilier et al., 1992; Christy and Dickman, 2002; Sanzo and Hecnar, 2006]. Some amphibians have evolved adaptations for brackish water containing high electrolyte concentrations [Katz, 1989; Gomez-Mestre and Tejedo, 2003] whereas others are intolerant and require fresh water with low levels of electrolytes [Smith et al., 2007]. The electrolyte concentration and balance of substrates is an area where species-specific ranges may need further investigation and refinement. In many species the skin contributes more sodium uptake than does the diet. In the case of potassium, diet is typically the primary source. Calcium is actively transported across the skin, thus water can be an important source for many species. Amphibians (primarily anurans) can store considerable amounts of calcium carbonate in their paravertebral lime sacs, which can be mobilized during periods of high demand such as metamorphosis or injury [Schlumberger and Burk, 1953; Pilkington and Simkiss, 1966; Stiffler, 1993; Wongdee and Charoenphandhu, 2013].

Observational evidence indicates that most species of amphibians do not drink water even when it is easily available; uptake of water occurs predominantly from cutaneous uptake together with input from preformed water in food and metabolic water production [Hillman et al., 2009]. Thus amphibians can have very high cutaneous evaporative water loss when humidity is low, and experience net water uptake when exposed to moisture. Cutaneous water uptake is especially important in many terrestrial species and is accomplished by the highly permeable skin in the ventral

pelvic region. Terrestrial anurans display a distinct cutaneous absorbing posture where their pelvic skin is pressed onto areas of high moisture or standing water for extended periods of time. Cutaneously absorbed water is processed by the kidneys and the hypotonic urine produced is stored in a large bladder attached to the cloaca. This stored water can be resorbed and used to defend against dehydration [Jorgensen, 1994; Hillman et al., 2009]. In addition to mechanisms of water uptake and storage, some anurans have reduced water evaporation through skin due to waxy secretions, enabling survival in arid and semi-arid conditions [e.g., Navas et al., 2004].

Nitrogen metabolism varies in amphibians with life stage and adult habitat, and nitrogen excretion strategy impacts the requirement for amino acids (in vertebrates that have been studied). Ammonia is metabolically inexpensive to excrete but very toxic and requires an aquatic environment for elimination, uric acid is expensive to synthesize but economical in terms of water needs for excretion, and urea is intermediate. Most amphibian larvae and aquatic adults are ammonotelic, while urea excretion is common in non-aquatic adults [Brown et al., 1959; Cragg et al., 1961; Atkinson, 1992]. Uric acid excretion is much less common in amphibians but occurs in several arboreal species that have skin that is very resistant to cutaneous water loss. Ammonotelic and uricotelic species lack an active urea cycle and thus have a relatively high dietary requirement for arginine, while the synthesis of uric acid increases the requirements for glycine and methyl groups originating from methionine. Although these nutritional relationships have not been well studied in amphibians, they should be considered when examining requirements because specific amino acid needs are likely to have a strong dependency on habitat and diet.

Malnutrition

A variety of disease conditions attributed to nutritional deficiency or excess have been reported in amphibians in breeding programs [Wright and Whitaker, 2001; Densmore and Green, 2007]. Vitamin deficiencies (particularly thiamin, but also other B-vitamins) have been proposed due to incidence of neurological and musculo-skeletal abnormalities associated with spindly leg syndrome and paralysis [Wright and Whitaker, 2001; Crawshaw, 2003]. The most important nutritional diseases recognized in recent years are associated with insect-based diets that have improper Ca:P ratios, and are generally deficient in fat-soluble vitamins such as vitamin A.

Vitamin A Deficiency

Vitamin A deficiency was first described as the "short tongue syndrome" (STS) in endangered Wyoming toads (*Anaxyrus baxteri*) that were part of a CBP [Pessier et al., 2005; Pessier, 2013]. Toads with STS would strike at prey items but were unable to prehend food giving the appearance of a shortened tongue. Histopathologic examination of the tongue from severely affected animals revealed replacement of the normal mucus-producing epithelium of the tongue with a keratinizing stratified squamous epithelium (squamous metaplasia). The observation of squamous metaplasia is a recognized indicator of vitamin A deficiency in mammals, birds and reptiles [Wolbach and Howe, 1925; Elkan and Zwart, 1967; Aye et al., 2000]. Subsequent investigation revealed very low liver retinol (vitamin A) concentrations in affected Wyoming toads. Clinical and pathologic findings suggestive of hypovitaminosis A have now been observed in a variety of ex situ amphibians including representatives of the families *Bufonidae*, *Dendrobatidae*, *Hylidae*, *Ranidae*, and *Rhacophoridae* [Sim et al., 2010; Pessier, 2013; Rodriguez and Pessier, 2014; this issue]. Because most ex situ amphibians are fed diets based on cultured insects (e.g., domestic crickets, *Acheta domesticus* and mealworms, *Tenebrio molitor*) that are generally poor sources of vitamin A, it is likely that vitamin A deficiencies are more common than currently suspected, and other consequences of hypovitaminosis A such as poor reproductive success, reduced survival of tadpoles, and immune system dysfunction could have significant impacts on the success of amphibian breeding programs.

There are no standardized criteria for the diagnosis of hypovitaminosis A in amphibians. In practice, combinations of clinical signs, histopathologic observation of squamous metaplasia in mucus-producing epithelial tissues, laboratory results such as liver or serum vitamin A levels and clinical response to vitamin A supplementation have all been used. Clinical signs such as difficulty in prehending prey (e.g., STS) are non-specific or are observed inconsistently because squamous metaplasia of the tongue is not found in all affected animals. The absence of squamous metaplasia does not exclude the possibility of preclinical hypovitaminosis A in any individual animal. Histopathologic evidence of squamous metaplasia in tissues collected at necropsy has been observed in a variety of anatomic locations, and could be missed if the pathologist is not well-versed in normal amphibian histology (e.g., mucus-producing epithelium of the tongue or columnar ciliated epithelium of the oral mucosa and esophagus). An overview of squamous metaplasia in cases of suspected hypovitaminosis A is provided in Rodriguez and Pessier [2014; this issue].

Currently, there are questions about interpreting blood and liver vitamin A data in amphibians to determine nutritional status. First, sufficient information is not presently available to establish "normal" ranges for serum vitamin A (retinol) in different amphibian species. It is known from studies in other animal species that serum retinol levels are maintained by hepatic total vitamin A (retinol + retinyl ester) stores. Therefore serum values may not accurately reflect vitamin A status, and hepatic vitamin A levels are the standard by which vitamin A status should be assessed [Bender, 2003]. In Wyoming toads the difference in mean liver retinol, determined by reverse phase HPLC at 325 nm, between STS-affected toads at 1.5 μg/g (range: not detected to 7.3 μg/g) and wild Wyoming toads at 105 μg/g (range: 44–164 μg/g) was striking. However, it is unclear if these findings are applicable across diverse amphibian taxa [Pessier, 2013]. Second, reported studies have not been clear in distinguishing if serum or liver vitamin A levels represent just retinol, or "total vitamin A" combining

both retinol and retinyl esters, and possibly even carotenoid levels, making it difficult to interpret the results. Third, reported studies have not clearly defined how blood/tissue samples were obtained, handled, processed and analyzed, and whether these were optimal for assuring the validity of the measures. Retinol and retinyl esters are biologically and chemically unstable and can be lost from samples if these are not handled appropriately. More detailed information on the subject is given by Clugston and Blaner [2014].

Treatment of suspected or confirmed hypovitaminosis A has been attempted using water-miscible formulations of vitamin A palmitate (Aquasol A, Mayne Pharma, Inc., Paramus, NJ) administered orally or by topical application to the skin. In addition, routine topical supplementation for at-risk animals has been adopted by some facilities and conservation programs. This is a costly and labor-intensive intervention, and has been viewed as a short-term solution by some population care-takers. Anecdotally, treatment has resulted in improvement in clinical signs, but experimental studies comparing oral and topical administration of water-miscible vitamin A formulations have shown mixed results in the ability to raise serum or liver vitamin A concentrations [Marin and Crump, 2010; Sim et al., 2010]. There are concerns about topical administration of vitamin A due to the highly variable skin of amphibians. Aquatic amphibians and those terrestrial species that live in moist environments have skin that is capable of high rates of cutaneous absorption of water and water-soluble substances [Quaranta et al., 2009]. Species living in more arid environments often have skin on most areas of their body that is less permeable than that of aquatic species but also have a highly absorptive patch of skin on their ventral pelvic region [Hillman et al., 2009]. Finally, some terrestrial species have skin that is highly resistant to cutaneous absorption. Thus, the dose of vitamin A absorbed across the skin is likely to be highly dependent upon the form of vitamin A (water soluble forms of vitamin A are typically chosen for application to mammalian skin), the type of skin the amphibian possesses, and the location on the body where applied. For this reason, dietary supplementation of vitamin A is likely to provide a more consistent and species independent route for dosing. Dietary supplementation of vitamin A may be accomplished with direct oral administration of vitamin A. Ideally, vitamin supplements for dusting or gut loading insect prey items should contain pre-formed vitamin A, beta-carotene, or pro-vitamin A carotenoids and should be stored under cool and dark conditions [Brenes and Dierenfeld, 2014; this issue; Li et al., 2009; Ogilvy et al., 2012a]. It is important to note, although not a significant concern, that hypervitaminosis A has been described in the literature; African clawed frogs are reported to be susceptible to high dietary vitamin A from mammalian liver and rodent prey items [Crawshaw, 2003], although this condition is probably unlikely with current feeding protocols.

It is clear that hypovitaminosis A is a serious concern in amphibian populations. Clarification of the vitamin A requirements for various species and life stages, consistent sampling and analytical protocols for establishment of reference ranges for "normal" individuals, and standardization of treatment of vitamin A deficiency is critical for the success of future efforts.

Metabolic Bone Disease

Metabolic bone diseases characterized by clinical findings such as weakness, tetany, poorly mineralized bones, and pathologic fractures, continue to be an important problem in amphibian populations. Skeletal deformities are especially common in juvenile animals of some species within Conservation Breeding Programs [Gagliardo et al., 2010]. In many cases the cause is inadequate supplementation of inherently calcium deficient insect-based diets, but in others there may be species specific needs for exposure to UV-B radiation in appropriate temperature ranges in order to meet vitamin D_3 requirements [Antwis and Browne, 2009; Antwis et al., 2014; Michaels et al., 2014]. Vitamin A deficiency has also been linked to metabolic bone disease in chameleons [*Chamaeleo calyptratus*; Hoby et al., 2010]. The role of water source and composition should not be underestimated in evaluating these conditions, with recent reports describing elevated phosphorus or fluoride levels in municipal water supplies as potentially contributory factors [Shaw et al., 2012; see Achieving Ecologically Appropriate Environmental Parameters: Light and Toxicology sections].

Recommended Nutrient Intake

Nutrient requirements for amphibian species are mostly unknown. Diversity of the three Orders within the Class *Amphibia* presents a challenge to make proper nutritional recommendations for this grouping of animals. The *Anura* order (frogs and toads) is the most fully studied, though select findings on *Caudata* (salamanders and newts) species are available. Also, the peculiar ontogenetic feeding strategies of the *Apoda/Gymnophiona* order (caecilians) have been recorded [Verdade et al., 2000; Gaborieau and Measey, 2004; Wilkinson et al., 2008]. In general, when recommending nutrient intakes for species with unknown requirements, the published nutrient requirements of related species are used (National Research Council, NRC). The choice of "related species" may be based upon environment, life stage, metabolism, or wild-type feeding habits. For amphibians, suggested species models for preliminary nutrient requirement recommendations are cats and dogs [NRC, 2006], which represent terrestrial-dwelling strict carnivores and omnivores, respectively, fish [NRC, 1993], which represent aquatic omnivorous and carnivorous species dwelling in many type of aquatic environments, poultry [NRC, 1994], which may provide insight on requirements for egg production and uric acid excretion, and rats [NRC, 1995], a basic terrestrial omnivore model. Integrating these NRC recommendations into a single set of nutrient recommendations for amphibians may provide valuable guidance for diet formulation of managed amphibians but also presents many challenges due to species differences. Table 1 presents some recommendations

TABLE 1. Preliminary recommendations for nutrient intake of amphibians post-metamorphosis, based on NRC recommendations for dogs and cats (NRC, 2006), fish (NRC, 1993), poultry (NRC, 1994), and rats (NRC, 1995)

	Amphibian recommendation (adult)[a]		Nutrient content of typical commercial feeder insects[b]					
Nutrient		Primary NRC model species	Adult crickets	Roaches	House flies	Mealworm larvae	Super-worms	Soldier fly larvae
Crude protein (%)[c]	44.4	Fish	58.5	47.4	85.8	36.4	32.5	35.1
Arginine (%)	2.6	Fish	3.6	3.5	5.3	1.9	1.6	2.5
Glycine (%)[d]	0.9	Poultry	3.0	3.1	3.7	2.0	1.6	1.8
Histidine (%)	0.8	Fish	1.4	1.4	2.5	1.2	1.0	1.2
Isoleucine (%)	1.2	Fish	2.7	1.9	3.5	1.8	1.5	1.5
Leucine (%)	1.9	Fish	5.9	3.0	5.4	3.9	3.2	2.4
Lysine (%)	2.6	Fish	3.1	3.2	5.5	2.0	1.7	2.4
Methionine (%)	0.8	Fish	0.9	0.8	2.5	0.5	0.3	0.7
Met + cysteine (%)	1.3	Fish	1.3	1.2	3.1	0.8	0.6	0.9
Phenylalanine (%)	1.1	Fish	1.9	1.9	3.4	1.3	1.1	1.5
Phe-tyrosine (%)	2.2	Fish	4.8	5.5	7.5	4.0	3.4	3.9
Threonine (%)	1.3	Fish	2.1	2.0	3.3	1.5	1.3	1.4
Tryptophan (%)	0.4	Fish	0.4	0.4	1.0	0.3	0.3	0.6
Valine (%)	1.4	Fish	3.1	3.1	4.8	2.1	1.7	2.6
Taurine (%)[e]	0.1	Cat	0.4	0.0	0.7	0.2	0.0	0.0
Crude fat (%)[f]			19.4	25.0	8.3	26.1	29.2	28.1
Linoleic acid (%)	[g]		6.5	5.4	1.8	6.8	5.4	3.4
Linolenic acid (%)	[g]		0.2	0.2	0.2	0.3	0.2	0.1
Arachidonic acid (%)	[g]		0.06	0.10	0.02	0.0	0.0	0.0
Calcium (%)	0.6	Rat	0.1	0.1	0.3	0.0	0.0	1.9
Phosphorus (%)	0.3	Rat	0.8	0.4	1.6	0.6	0.4	0.0
Sodium (%)	0.2	Cat	0.4	0.2	0.6	0.1	0.1	0.0
Magnesium (%)	0.04	Cat	0.09	0.06	0.35	0.16	0.08	0.00
Potassium (%)	0.4	Cat	1.0	0.6	1.3	0.7	0.5	0.0
Chloride (%)	0.1	Cat	0.7	0.4	0.8	0.4	0.3	0.2
Copper (ppm)	12	Dog	17.7	19.7	56.2	11.9	5.9	8.0
Iodine (ppm)	1	Fish	0.6	0.7	0.0	0.3	0.0	0.5
Iron (ppm)[h]	97	Poultry	55.1	37.0	544.7	40.1	27.2	133.6
Manganese (ppm)	14	Fish	32.8	6.5	115.9	10.1	7.1	124.0
Selenium (ppm)[i]	0.3	Cat	0.5	0.7	6.5	0.5	0.2	0.6
Zinc (ppm)	18	Fish	191.4	81.6	373.9	101.2	50.7	112.7
Ascorbic acid (ppm)[j]	23	Fish	85.6	0.0	0.0	23.3	19.8	0.0
Biotin (ppm)	1	Fish	0.5	0.9	3.0	0.6	0.6	0.7
Choline (ppm)	1,889	Dog	4,334	2,017	2,471	3,588	2,866	2,207
Folic acid (ppm)	1	Fish	4.3	2.8	7.9	3.1	1.1	5.4
Niacin (ppm)	44	Cat	109.6	109.4	394.3	79.2	53.3	142.4
Pantothenate (ppm)	23	Fish	65.6	92.4	194.4	51.0	32.0	77.2
Pyridoxine (ppm)	7	Fish	6.6	7.7	7.4	15.8	5.3	12.0
Riboflavin (ppm)	8	Fish	97.3	39.0	336.4	15.8	12.4	32.5
Thiamin (ppm)	12	Fish	1.1	2.2	49.2	4.7	1.0	15.4
Vitamin B_{12} (μg/kg)	39	Dog	153.2	591.8	26.1	9.1	6.9	111.9
Vitamin A (IU/kg)[k]	2,914	Fish	0.0	0.0	0.0	0.0	0.0	0.0
Vitamin D3 (IU/kg)[l]	1,111	Rat	0.0	482	434	0.0	0.0	200
Vitamin E (IU/kg)[m]	88	Fish	56.2	0.0	192.6	0.0	12.7	18.5
Vitamin K (ppm)	2	Dog	NA[n]	NA	NA	NA	NA	NA

[a]All recommendations are based on a diet containing 4,000 kcal DE/kg, and on a dry matter basis (DMB). These recommendations were created by tabulating the recommendations for the species noted above, and then (1) using the recommendation for the most appropriate species if apparent, (2) using the median recommended intake if no species model was notably appropriate, (3) using the highest or lowest recommended intake of all species if deficiency or toxicity were a concern. [b]All nutrient values are standardized to 4,000 kcal DE/kg, and on a dry matter basis (DMB). [c]Higher protein intakes associated with reduced developmental abnormalities (Martinez et al., 1994; Venesky et al., 2012; Martins et al., 2013). [d]Essential for uric acid production. [e]Limited data on essentiality of taurine in amphibians. [f]Excess dietary fat may result in food intake limitations, which may reduce intake of other nutrients. [g]Recommendations for linoleic, linolenic, arachidonic acid, EPA, and DHA are not made at this time due to substantial variability in the recommendations for other species, but are likely all essential. Additionally, levels of some fatty acids in insects may be modified due to their diet composition during growth and development. [h]Nucleated red blood cells. [i]Risk of toxicity. [j]Ability to synthesize not documented in all amphibians. [k]Appropriate form and source of vitamin A still being examined. Coated pre-formed vitamin A may not be available for consumption by insects due to particle size (Attard, 2013). [l]UV-B exposure has been shown to impact bone mineralization [Verschooren et al., 2011], and amphibians have capacity for endogenous synthesis of vitamin D_3 [Holick, 1995; Michaels et al., 2014]. [m]Recommendation proportional to omega-3 content of diet. [n]NA, not analyzed.

for nutrient intake of amphibians' post-metamorphosis, along with a comparison of nutrient content of several common commercial feeder insects. These values likely represent adequate amounts rather than true minimum requirements, which cannot be established without empirical research trials.

Nutrients for which recommendations were not made, but are likely to be important in amphibian nutrition include linoleic acid, linolenic acid, arachidonic acid, EPA and DHA, for which considerable diversity in recommendations for other species exist, as well as inositol and fluoride [Zhao et al., 2013]. Additionally, carotenoids have received recent attention for their role in pigmentation, vitamin A status, and reproductive success [McComb, 2010; Ogilvy et al., 2012a,b; Finke, 2013; Brenes and Dierenfeld, 2014; this issue], but information is insufficient for making recommendations at this time.

The role of gastrointestinal microflora on amphibian nutrition is still relatively unclear. Recent work has documented gut microbial communities of salamanders [Okelley et al., 2010] and frogs [Kohl et al., 2013], and has shown that gut microflora varies due to life stage [Kohl et al., 2013] and environmental temperature [Gossling et al., 1982; Banas et al., 1988; Woodhams et al., 2008]. Efforts to utilize probiotics (via water or soil inoculation) are underway to improve amphibian nutrition and health, in particular to mitigate effects of chytridiomycosis [Bletz et al., 2013].

Nutrition Research Models

Research models available to study nutrition of managed amphibians are limited due to the broad range of habitats, life stages, and feeding strategies that exist for amphibians. However, data are available from amphibian species in commercial production (e.g., bullfrog *Lithobates catesbeiana* [Carmona-Osalde et al., 1996; Olvera-Novoa et al., 2007]; Perez's frog *Lithobates perezi Sloane* [Martínez et al., 1994; Martinez et al., 2004]; Fowler's toads *Bufo woodhousei* [Claussen and Layne, 1983]; and European common frog *Rana temporaria* [Miles et al., 2004]). Data are also available from amphibia managed in laboratory settings, in particular, *Xenopus laevis* [Brown and Rosati, 1997]. Additionally, availability of the *Xenopus laevis* genome (http://www.xenbase.org/genomes/static/laevis.jsp) may enhance the ability to study expression of nutrient transporters and other information at the genome level.

Food Items for Ex-Situ Amphibian Populations: Nutritional Composition of Feeder Insects

Unlike their ex situ counterparts, most wild insectivorous amphibians feed on a variety of invertebrates, with quite variable nutrient composition. In addition, the food within the invertebrates' gastrointestinal tract and the material clinging to their exoskeleton (such as soil and pollen) also adds to the variety of nutrients consumed by wild insectivores. In contrast, ex situ managed insectivorous amphibians are fed a much smaller variety of commercial feeder insects and other invertebrates (less than 20–30, and likely smaller numbers available consistently). A recent survey [Sincage, 2012] answered by 212 institutions in the United States and Europe shows most institutions rely heavily on crickets (*Acheta domesticus*) as the main diet item but yellow mealworm larvae (*Tenebrio molitor*), superworm larvae (*Zophobas morio*), waxworm larvae (*Galleria mellonela*), fruit flies (*Drosophila melanogaster* and *hydei*), roaches (*Blaberus* and *Gromphadorhina* spp), and various species of Annelids (worms) are also regularly used to feed most managed insectivorous amphibians. In addition to their nutrient content, behavioral criteria also play a role in the proper selection of feeder insects. Mealworm and waxworm larvae are not particularly mobile so they may not be the best choice for insectivores stimulated to feed by movement. Additionally some species are not particularly good at clinging to vegetation and hence may not be appropriate for arboreal species. Finally, some insect species are of limited availability in certain regions; United States federal regulations (as well as those of other countries) prohibit the inter-state transportation of many potential species of feeder insects due to concerns of them becoming agricultural pests if they escape.

Comparisons between nutrient content of wild and commercially available insects are limited. Complete nutrient profiles (minerals, vitamins, amino acids, and fatty acids) are now available for many common feeder insects [Jones et al., 1968; Barker et al., 1998; Finke, 2002; Oonincx and Dierenfeld, 2011; Finke, 2013]. Data for wild insects consists of moisture, protein, fat, ash, fiber, fatty acids and select minerals [Reichle et al., 1969; Levy and Cromroy, 1972; Studier and Sevick, 1992; Studier et al., 1992; Punzo, 2003]. Amino acid and vitamin concentrations of a limited number of wild insect species have been documented [Pennino et al., 1991; Ramsay and Houston, 2003; Rumpold and Schlüter, 2013].

Based on published data, in general, commercially available insects appear to be good sources of protein, amino acids, most B-vitamins and most minerals (except calcium). A diet consisting primarily of insect larvae may be borderline deficient for some of these nutrients due to caloric dilution since insect larvae are typically high in energy [Finke, 2002]. For insectivorous amphibian diets composed of a variety of commercially available insects, nutrients of potential concern are calcium and the vitamins A, D & E and thiamin (Table 1, and see Malnutrition section). While managed amphibians appear to meet their vitamin D requirements through appropriate lighting [e.g., Verschooren et al., 2011], dietary vitamin A and calcium supplementation is required. Few insects have a calcified exoskeleton; those that do may provide a viable source of calcium for insectivores. One example is soldier fly larvae (*Hermetia illucens*), which are available commercially, but some caution should be taken as some animals have shown difficulties in digesting the larvae [Dierenfeld and King, 2008]. Another high-calcium invertebrate that should be considered for feeding programs is the pillbug or wood louse [*Porcilio scaber*; Oonincx and Dierenfeld, 2011]. Most commercially available insects also contain little to no carotenoids relative to wild caught

insects [Eeva et al., 2010; Arnold et al., 2011; Newbury et al., 2013]. Carotenoids have been reported to aid in the coloration and health of amphibians although it is unclear if they can serve as a precursor for vitamin A/retinol [Ogilvy et al., 2012a,b; Dugas et al., 2013; Brenes and Dierenfeld, 2014; this issue].

Nutrients expected to be present at low levels in insects can be enhanced to increase their levels prior to feeding to insectivores. Two commonly used methods are gut loading and dusting [Livingston et al., 2014]. Gut loading describes the feeding of a special diet to insects just prior to the insects being consumed such that the diet will be present in the insect gut when consumed by the insectivore. Gut loading is suitable for most nutrients as long as the diet is palatable to the insect, the form of the nutrient is easily consumed by the insect and the diet contains sufficient quantities of the desired nutrient(s). Most research on the effects of gut loading has focused on increasing the calcium content of insects although vitamin A and some other nutrients have been studied [Strzelegicz et al., 1985; Allen and Oftedal, 1989; Pennino et al., 1991; Anderson, 2000; Klasing et al., 2000; Finke, 2003; Hunt-Coslik et al., 2009; McComb, 2010; Oonincx and Van der Poel, 2010; Ogilvy et al., 2012a,b; Attard, 2013]. Gut loading has been shown to be effective in a large number of insect species and the calcium from gut loaded yellow mealworms was shown to be readily available to growing chicks [Klasing et al., 2000]. Optimal gut loading time varies, possibly due to the insect species being studied, palatability of the gut loading diet, and environmental conditions (temperature, light and humidity). In general, gut loading for a period of 24–72 hr appears to result in similar levels of nutrients in the intact insect. When high calcium gut loading diets are fed for longer periods of time adverse effects on insect viability have been observed [Klasing et al., 2000].

Dusting is the term used for coating an insect with a fine powder containing the desired nutrients, such that the powder adheres to the outside of the insect. An important factor in the effectiveness of this method is the time between dusting and consumption [Trusk and Crissey, 1987; Sullivan et al., 2009]. House crickets can groom off up to half of the amount of adhering powder in 90 sec [Li et al., 2009]. It is also difficult to estimate the amount of dust adhering to the insect, which is affected by the physical characteristics of the diet, insects' exoskeleton, and relative surface area of the insect. Dusting insects in environments with high humidity is not suitable to enhance nutrient content nor for aquatic insectivores.

An additional level of nutrient modulation may be achieved during the growing period of domestically reared insects; insect composition can be altered by both environmental conditions (light, temperature, and photoperiod) as well as by diet [Schaefer, 1968; Kaplan et al., 1986; Sonmez and Gulel, 2008]. Recently, crickets, mealworms, and superworms with enhanced levels of beta-carotene, vitamin E, and omega-3 fatty acids have become commercially available (e.g., Vitabugs™ Timberline Live Pet Food, Marion, IL).

Food Items for Ex-Situ Amphibian Populations: Safety and Quality of Feeder Insects

There are no generally accepted guidelines for determining the quality of commercial feeder insects other than that they are alive and hence a viable food source. Insect producers, feed manufacturers, and facilities managing insects prior to their use as prey items should consider:

- Avoidance of antibiotics or other growth promoters in diets for insects.
- Prevention of mycotoxin contamination of diets for insects. Although insect species seem to be relatively resistant to moderate levels of aflatoxin [McMillian et al., 1981; Dowd, 1992; Trienens and Rohlfs, 2012], amphibians are likely adversely affected by mycotoxins similar to their well-known effects on fish, birds and mammals.
- Prevention of pathogenic bacteria and viruses in insects and feed. While *Salmonella* is not a major clinical issue in managed amphibians, insects may be vectors for *Salmonella* spp. both externally and internally [Crippen et al., 2009]. Insect specific pathogens (e.g., *Acheta domesticus* densovirus or cricket paralysis virus, invertebrate iridoviruses) are also of concern and while not all species are equally susceptible they may serve as a vector for the virus to move into a susceptible population [Weinmann et al., 2007; Szelei et al., 2011]. To minimize this risk, producers should ensure their diets are free of pathogenic bacteria, and that feeder insects are maintained under hygienic conditions with standard biosecurity practices [Pessier and Mendelson, 2010].

Issues in Ex Situ Husbandry

While amphibians have been the subject of observation, breeding, and research in ex situ populations for decades, in more recent years there has been a nearly exponential increase in the numbers of amphibians brought into ex situ programs for conservation purposes. With the increase in interest in CBPs comes a need to improve the efficiency of keeping large numbers of animals. Often husbandry techniques and other aspects that contribute to animal health are affected by the schedules and needs of their keepers rather than what is best for the animals in their care.

There are many basic resources available to amphibian caretakers through organizations such as Amphibian Ark (AArk), the Association of Zoos and Aquariums (AZA), scientific journals, and countless Internet resources [e.g., Zippel et al., 2006]. The AZA Husbandry Resource Manual [Pramuk and Gagliardo, 2012] and the Conservation Breeding Specialist Group Disease Manual [Pessier and Mendelson, 2010] provide adequate information for basic amphibian care and important guidelines on biosecurity and disease issues. For each focal taxon involved in Species Survival Plans (SSPs), or those species with formalized and cooperative breeding and management plans, the AZA Amphibian Taxon Advisory Group has developed specific

husbandry manuals to help guide caretakers in maintaining these species.

Natural History Reference Values for Each Life Stage

Amphibian caretakers frequently find themselves attempting to mimic nature in providing adequate conditions for amphibians in their care. More often than not, due to limited resources of space, time, and funding this is not possible. In addition, while there is descriptive information available on the behavior of some amphibians in nature [Stebbins and Cohen, 1995], there is a general lack of natural history-derived reference values. Another complicating factor is that there are multiple life stages to consider, particularly in active breeding programs. Collecting and applying information obtained from observations in nature or directly from physical specimens (museum specimens included) can be extremely useful and there is a need for this general information to be collected in a standardized database [specific recommendations are listed in Olea-Popelka et al., 2014] with this information compared for all life stages of an individual species or across genera.

Achieving Ecologically Appropriate Environmental Parameters: Water Quality

Water quality may be one of the most important and sensitive parameters in amphibian husbandry. The best method for obtaining ecologically appropriate water quality is to utilize a natural water source in an enclosure through an open system. This method, however, is relatively rare in practice and has inherent risks. One example is applied in the Japanese giant salamander or hanzaki (*Andrias japonicas*) breeding program at the Asa Zoo in Hiroshima, Japan [Gagliardo, 2013, personal communication]. Since the zoo is located in the salamander's natural habitat, it affords the ability to route water from nearby streams through the zoo breeding enclosures, thus providing similar water quality conditions to those present for salamanders in the wild. However, this method also has risks in exposing animals to environmental pathogens, infectious diseases, and other potential contaminants such as pesticides and chemicals.

The more common type of open system is an artificial one whereby another source of water, such as potable water, collected rainwater, or reverse osmosis (RO) water, is used after treatment. While this method is not considered environmentally efficient due to production, transport, and disposal energy costs, it does afford the most control over removing and preventing waste products in the exhibit. Artificial open systems require the treatment of the source water and strict monitoring of water quality parameters such as chlorine, chloramines, hardness, pH, dissolved oxygen, and total dissolved gas pressures. Treatment options such as activated carbon filters or reverse osmosis units have proven to provide good methods of addressing chlorine and chloramines issues depending on the incoming source water [Russell, 2007]. Chlorine can be detrimental to an RO unit's membrane; therefore it is highly important to understand the chemical makeup of the source water and determine the ideal treatment method prior to implementation [Glater et al., 1994; Shintani et al., 2007]. De-gas towers or waterfall features assist with creating water turbulence and equilibrate the dissolved gases in the water with the atmosphere solving low dissolved oxygen levels or high total dissolved gas levels in source water. The pH of source water is also an important factor, as most potable water is slightly basic; whereas collected rainwater is expected to have a more acidic pH. Treatment for pH depends on the species' natural environment, as amphibians have diverse acceptable pH ranges [Odum and Zippel, 2008; Odum and Zippel, 2011]. Potable water may also be higher in calcium, magnesium, and boron, depending on where the treated water originated [WHO, 2011]. Hard water can also cause scaling on exhibit plumbing and result in cloudy water, particularly if paired with an elevated alkalinity.

While open systems provide a constant method of waste removal, these types of systems may not be practical. Closed systems can provide more control and balance of water quality parameters if managed properly; however, regular testing for critical parameters along with total alkalinity, temperature, ammonia, and nitrite should occur to make sure the system's components are functioning properly [Pramuk and Gagliardo, 2012]. A closed system should contain mechanical and biological filtration, a method of gas exchange with the atmosphere, temperature control (if needed) and possibly a method of disinfection. System turnover (100% exchange) is one of the most important factors when designing or selecting an appropriate life support system. Good turnover rates limit problems associated with maintaining water quality, cycling nitrogenous wastes, and allow constant gas exchange. Total dissolved gas pressures should be monitored frequently as the life support system components, such as pumps, can trap air in the water and cause an increase in total dissolved gases in the exhibit [Pramuk and Gagliardo, 2012].

Establishing a healthy biofilter is one of the most important components of a closed aquatic animal habitat especially considering nitrite concentration's effect on larval amphibians [Odum and Zippel, 2011]. Concentrated nitrifying bacteria can be purchased and dosed into the Life Support System to colonize appropriate surfaces and establish the nitrification process (FritzZyme, FritzZyme Industries, Dallas, TX); however, it is important to take any disinfection systems offline prior to dosing the system and ensure that the chemical and physical properties of the water are ideal for the bacteria. While nitrification most likely occurs anywhere there is surface area for the bacteria to colonize, the majority occurs in the mechanical filter; disrupting this filter could in fact disrupt the biological filter as well [Michaud et al., 2006].

Recently, ecological filtration methods that harness algae to remove trace metals, phosphates, and nitrogenous wastes from the aquatic system and also assist in the addition of dissolved oxygen to the system are being employed. These

methods are more ecologically balanced and rely on natural processes of removing wastes, which more likely mirror a natural environment [Odum and Zippel, 2008].

Achieving Ecologically Appropriate Environmental Parameters: Light

One way to provide appropriate lighting systems for ex situ amphibians is to identify the critical parameters found in the species' natural habitat and apply natural and/or artificial treatment methods to manage those parameters within acceptable ranges, which can be difficult to accomplish. Regardless of natural light levels, amphibians need to be offered light, along with a complete refuge away from light, that is, accessible at all times. Most species need lighting gradient options to self-adjust to suitable output levels, as some can be negatively affected by exposure to UV-B light. Amphibian skin is much thinner than reptile skin, with the epidermis normally 2–5 cell layers thick [Farquhar and Palade, 1964]. As a result, the effects of damage to amphibian skin can result in serious conditions. A field study concluded that the levels of ultraviolet, or UV-B, radiation found in sunlight can cause physical deformities in amphibians [Blaustein et al., 1997]. Amphibian eyes are also very vulnerable to excess UV-B. Experimental effects of UV radiation (280–315 nm) on both tadpole and adults stages of Pacific tree frogs (*Hyla regilla*) and red-legged frogs (*Rana aurora*) resulted in the development of cataracts [Adkins et al., 2003]. These eye issues occur in nature due to habitat destruction, and also occur in ex situ populations as a result of inadequate lighting gradients. The effects of desiccating, high light, and strong UV-B environments are species specific, as some amphibians may exhibit natural behaviors that expose them to drying environments and high UV-B.

While spectral requirements for amphibians are vague or unknown, most literature suggests the use of ultraviolet lighting [Adkins et al., 2003]. Ultraviolet light deficiency can result in poor feeding, muscle weakness and tremors, lack of vibrancy or vigor, poor growth and reproductive history, abnormal posture, paradoxical soft tissue mineralization, hypotonia, lameness, and metabolic bone disease and its associated conditions [Adkins et al., 2003; Browne et al., 2009]. Many of these conditions are common in ex situ amphibians, but it is difficult to separate clinical signs between dietary imbalances and UV light deficiency. Little is known about the actual daily needs with regards to exposure to UV-B, and caution should be used in exposure in the laboratory. The ultimate goal is to tailor a unique lighting management strategy for each niche type utilizing modern lighting options, safety light gradients, and shade options, which are similar to the animals' natural habitat [Michaels et al., 2014].

Perhaps the most attractive option with lighting is to meet the animals' lighting requirements with natural sunlight in the ex situ environment, which can be accomplished in outdoor enclosures with minimal cost and effort. In recent years, amphibian population managers have developed unique light-channeling devices to direct and control UV-B light exposure and/or full-spectrum solar light for their animals [e.g., UV-channeling SunPipe system, Sunpipe Co., Inc., Batavia, IL; K. Semmen, unpub. data]. Several companies (Himawari, Parans, Sunlight Direct) have solar lighting fiber optic systems available, which use rooftop collectors such as a solar tracking device to gather sunlight, and a fiber optic cable bundle to carry the light into the building. Although these systems are designed to filter out UV-light, UV-transmitting fiber optic cables are available as well.

Supplementation of natural lighting with artificial lighting is likely the easiest way to ensure proper lighting spectra and intensity, especially regarding proper UV-B lighting. There are many sources of artificial UV light available [Gerhmann, 1987] to supplement natural UV. It is important to choose the right source of artificial UV and make use of quality analytical equipment to routinely measure and manage the proper intensity of UV-B of the appropriate wavelengths (290–310 nm) in the proper amounts. Artificial sources of UV-B include the UV-B fluorescent tube and the mercury vapor (MV) reptile lamps. UV-B light emitting diodes (LEDs) in the required wavelengths exist, but they are currently (at the time of this paper) too low-energy and high-priced to be practical. Both fluorescent and MV styles use the effect of an electric arc through mercury vapor to produce ultra-violet radiation. Each style produces different amounts of total UV-B as well as useable UV-B. Quality MV lamps produce more UV-B in the critical range than even the best fluorescents at much greater distances. Both types suffer UV-B decay over time, which should be tracked with analytical testing, or if this is not performed, lamps should be replaced at least every 6 months. Typically an ultraviolet radiometer (i.e., Solarmeter Model 6.2 UV-B, Solartech, Inc., Harrison Township, MI), or spectrometer (i.e., Ocean Optics USB-2000, Ocean Optics, Dunedin, FL) is used for tracking UV-B light output over time. Ideally, iso-irradiance charts ("spread charts") should be developed for each enclosure to track output at predetermined distances and shapes from the light source on a routine basis as part of the quality control program to track artificial light as well as natural sunlight angles and intensity changes. The beam must be of the right shape where the animal is able to place most, if not all of its body within the zone of effective UV-B radiation at a suitable basking distance, and UV-B danger zones should be identified and made inaccessible to the animals.

With many amphibian enclosures located in the interior of building spaces and the potential constraints with using solar light, using artificial lighting is often the only practical option. This puts the emphasis on the advantages of artificial lighting economy, design simplicity, and parameter control. Fortunately, with modern automation control and LED lighting banks combined with supplemental UV lighting, there are few limits when it comes to duplicating diurnal and seasonal solar radiation patterns for ex situ housing. Strategically placed light sensors communicating back to a central supervisory control and data acquisition (SCADA)

system can complete the design for an intelligent lighting system that can compensate for aging lamps or provide dynamic basking sites. When these intelligent systems are used with both artificial and natural light options, artificial light can be automatically ramped up or down to augment fluctuating solar light levels due to cloud cover or seasonal changes in the daily amount and angle of sunlight. Lighting is critical to amphibian management, and with the many options, species-specific standards should be developed where possible, and documentation of successes and failures should be widely shared.

Chronic Stress

Numerous studies have clearly described the stress caused to wild amphibian populations by chemical contamination, disease, climate, and population density [Davidson and Knapp, 2007; Wilmers et al., 2007; Blaustein et al., 2012]. Addressing chronic stress in ex situ settings involves a detailed examination of overcrowding, species social structure, groupings in enclosures, and the ability of animals to use refugia and visual barriers, which may prevent stress from exhibit-mates or those in adjacent enclosures. Other aspects not often considered as adding stress to individuals are the effects of external stimuli such as vibration, sound, light (including the implementation of abnormal circadian rhythms), and frequent handling, as well as a lack of stimuli resulting in boredom or lack of any social interactions [Heatwole and Sullivan, 1994]. While one could argue that similar stresses are present in natural environments [Alford and Richards, 1999; Pounds et al., 2006], animals have the ability to move away from many of these stressors in nature. In the confines of an ex situ environment, even the simplest of stress factors may affect the welfare of the animal. Measuring this stress may be partially addressed by monitoring hematological data [Davis et al., 2008; Davis and Maerz, 2011]. However, one other aspect to consider is that simply handling the animal to retrieve samples for evaluating stress can also trigger stress [Langkilde and Shine, 2006]. Recent advancements in the use of urine sampling to non-invasively monitor stress hormone physiology in amphibians [Narayan, 2013] may provide additional opportunities. Since stress can have long-term effects on population health and reproduction, researchers and animal husbandry personnel should consider studies utilizing urinary stress hormone monitoring to determine what conditions result in stress responses in their species of interest, and focus on minimizing its effect [see Stress Endocrinology section; Moore and Jessop, 2003].

Maintaining Physical Condition

Creating complex environments based on natural history helps animals maintain better physical condition. This includes proper enclosure furnishings such as perches and climbing opportunities (via living or artificial plants, branches, etc.) and burrowing opportunities for more terrestrial species. These complex environments can be used to promote natural behaviors. For example, periodically offering a nutritious but smaller food item, that is, more physically challenging to find and consume can be used to promote foraging and is one type of enrichment caretakers can offer. In addition, the possible effects of seasonality should also be considered in maintaining body condition of amphibians. Measuring the overall fitness of an ex situ amphibian population may vary by species and should be as objective as possible. Obesity is a common problem observed in captive amphibians, which generally results from a combination of inactivity and poor diet (see Issues in Nutrition section). More careful attention to husbandry aspects such as enclosure design, social groupings and interactions, and feeding techniques will help to avoid not only obesity issues but will increase overall health and reproductive potential of ex situ amphibians [Browne and Zippel, 2007].

Husbandry-Related Disease

Skin Disease

The skin of amphibians is highly permeable and has unique physiologic functions including water and electrolyte exchange and in some species, respiration. These functions, combined with a lack of protective features such as a thick corneal (keratinized) layer, hair, or scales, make amphibians uniquely susceptible to systemic effects of even relatively minor skin disease when compared to other vertebrates [Crawshaw et al., 2014; this issue]. An important example of the potential impact of skin disease on electrolyte balance is illustrated by experimental studies of the fungal disease chytridiomycosis [Voyles et al., 2009].

Review of clinical and necropsy data from SSPs for the Puerto Rican crested toad (PRCT) and Wyoming toads (WT) as well as other amphibian conservation programs has shown a high prevalence of skin disease characterized histologically by varying degrees of epidermal hyperplasia and hyperkeratosis. In the PRCT the condition has been termed "brown skin syndrome" and is considered to be a dysecysis [abnormal skin shedding; Crawshaw et al., 2014; this issue]. Concurrent bacterial and fungal infections can be observed but are likely secondary or opportunistic because of a multifocal distribution rather than being present diffusely throughout the lesions. By themselves the histologic findings are non-specific, and overlap with lesions that can be seen secondary to a variety of skin injuries or primary disease conditions. For example, skin irritation due to water quality problems (e.g., elevated ammonia) is frequently identified in association with epidermal hyperplasia and hyperkeratosis. In the affected PRCT and WT, specific water quality issues have not been identified, which has led to a variety of hypotheses including the possibility of nutritional or environmental co-factors. Husbandry and disease in amphibian skin diseases are interrelated, and cooperation between amphibian keepers and veterinarians is necessary to understand the effects of environment on these diseases.

Kidney Disease

A common clinical presentation in amphibians is "edema syndrome" characterized by fluid accumulation in the subcutaneous lymph sacs and coelomic cavity [Densmore and Green, 2007]. The differential diagnosis is broad and ranges from infectious disease (e.g., bacteremia and *Ranavirus* infection) to husbandry factors (water with low solute concentration) to organ failure. Although antemortem diagnosis is difficult, review of necropsy findings shows a high prevalence of kidney disease in affected individuals [Mangus et al., 2008; Pessier, 2009]. Multiple disease processes are likely to be occurring based on the wide spectrum of renal tubular and glomerular lesions that have been observed by histopathology. To date, little has been done to describe and classify lesions thoroughly or conclusively determine potential etiologies. Like the non-specific skin disease syndromes mentioned above, differentials for degenerative and polycystic renal tubular lesions observed in species such as the Wyoming toad and Panamanian golden frog (*Atelopus zeteki*) could include husbandry-related factors such as water composition, environmental parameters, and nutrition.

General Biology and Research

Using a scientific or research framework to answer questions about disease, nutrition, or husbandry of ex situ amphibian populations has already led to improvement in amphibian specialists' understanding of the needs of these species. Despite the wealth of information gathered to this point, more questions remain, and some scientific disciplines are underutilized to address these questions.

Population Genetics

From the perspective of population genetics, the most critical point of ex situ population management is to maintain genetic diversity reflective of the natural population, so that the ex situ population will not lose genetic material and will maintain the potential to adapt to a changing environment [Fernández and Cabellero, 2001]. Amphibian populations are known to have very small effective population sizes, which in turn makes them susceptible to loss of genetic diversity by random drift, and particularly to the effects of inbreeding depression and a high genetic load [Funk et al., 1999; Frankham et al., 2002; Rowe and Beebee, 2003]. Loss of genetic diversity can lead to reduced reproductive success and decrease the probability of persistence of the ex situ population. Effects of inbreeding depression include lower clutch sizes, reduced hatchability and metamorph production, and susceptibility to disease [Rowe and Beebee, 2003; Halverson et al., 2006].

Ex situ populations should be initiated and managed with respect to best practices for maintaining genetic diversity which are critical to planning and developing ex situ conservation action [Mace, 2004]. By definition, population sizes in the wild need to be large to be viable [Gilpin and Soule, 1986], and thus when founding an ex situ population, the founding individuals ideally should represent all the genetic material from the population of which they were sampled, with at least 20 unrelated founders needed to capture this gene diversity [Lacy, 1989; Lacy, 1994]. However, founding populations may not be as diverse as assumed, with typical methods of collecting founders leading to the gathering of individuals from family groups, rather than completely unrelated individuals [Haig et al., 1995]. Small populations are also more likely to accumulate more deleterious mutations [Lynch et al., 1995; Wang et al., 1999]. Understanding the relationships between individuals enables researchers to calculate inbreeding levels, which allows for the investigation into whether biological parameters, such as clutch size or hatchability, are being affected by inbreeding depression [Hedrick and Kalinowski, 2000] and the likelihood of sharing deleterious recessive alleles [Lynch et al., 1995]. With known relationships, researchers can also examine specific genes, which are under selection, in order to evaluate local adaptation or even a population's susceptibility to disease. For example, certain allele frequency combinations of the highly polymorphic major histocompatability complex (MHC), a suite of genes associated with controlling immune function, were shown to predict better survival from chytrid fungus in the lowland leopard frog [*Lithobates yavapaiensis*; Savage and Zamudio, 2011].

Inclusion of molecular level population genetic data can address many specific questions when establishing an ex situ amphibian population, as well as improvement of management decisions related to maintaining and maximizing genetic diversity. Within the amphibian species that are currently managed collectively by institutions accredited by the AZA, there is a range of how much molecular genetic data have been included in the management of species to date. For example, both the Wyoming Toad SSP and Mississippi or Dusky Gopher Frogs (*Rana sevosa*) SSP have utilized a cursory evaluation of genetic diversity [Richter et al., 2009; Martin, 2010; Martin et al., 2012], but thus far this research has not been applied to management of the ex situ populations. The Houston Toad (*Anaxyrus houstonensis*) and Puerto Rican Crested Toad SSPs have incorporated molecular genetic research into their management. In these cases, each SSP has identified different ways that the genetic research goals can contribute to the ex situ management [Beauclerc et al., 2010; Barber et al., 2011; Muñiz, 2011; Vandewege, 2011; Crump and Schad, 2013]. However, there is a need for more specific research questions in amphibian population genetics, related specifically to how to improve the management of these highly fecund species with short generation times, how best to manage for conservation goals, how to integrate emerging molecular and cryopreservation techniques into management [Kouba et al., 2013] as well as how genetics (e.g., inbreeding depression, loss of alleles) is tied to overall amphibian population health [Schad, 2008].

Toxicology

While toxicological studies intended for in situ amphibian conservation initiatives abound, reports of ex situ toxicological issues outside of routine husbandry concerns are relatively rare. In terms of nutrition-related toxicological concerns, amphibian nutrition guidelines (see Issues in Nutrition section) should be followed to verify safety and quality of food items. Following recommended nutrient levels (Table 1) would minimize concerns regarding over-supplementation and associated toxicity. Amphibian husbandry guidelines [see Issues in Ex Situ Husbandry section; Poole and Grow, 2012] should be consulted to ensure proper cleaning protocols are in place (for both foodstuffs and habitats). These guidelines [e.g., Issues in Ex Situ Husbandry, above; Odum and Zippel, 2011; Poole and Grow, 2012] have also outlined appropriate and inappropriate materials used for housing and managing amphibian collections. The amphibian water quality guidelines [e.g., Odum and Zippel, 2011; Poole and Grow, 2012] include appropriate targets and limits for potential toxicants such as chlorine, ammonia, phosphate, carbon dioxide, and other water quality levels and should be used to avoid amphibian exposure to toxic levels. In terms of husbandry and water quality, fluorosis was cited as a factor in the development of metabolic bone disease in a frog species [Shaw et al., 2012].

Ecology and Behavior

In general, there is a lack of baseline data and comparative information for most basic ecological aspects of amphibian biology, particularly of those species that are managed in ex situ collections or those that have been identified as declining or of conservation concern (see Issues in Husbandry section). While ecological aspects of many non-threatened and widespread species have been studied extensively, often those species near extinction are also those most under-studied. In addition, much research in the field is focused heavily on documented causes of decline of amphibian populations [Beebee and Griffiths, 2005]. Clearly, there is a need for more basic data on life-history parameters, local adaptations, habitat use, dispersal behavior, and population dynamics, especially factors influencing long-term persistence of amphibian species and their habitat [Semlitsch, 2002; Young et al., 2001]. However, because some species are in urgent need of conservation action, we cannot afford to wait for additional data from wild populations, and thus ex situ populations can serve as controlled study systems to answer some of these basic biological questions [Semlitsch, 2002].

Stress Endocrinology

Historically, studies examining endocrine function in amphibians have focused primarily on monitoring or inducing reproduction and the study of growth and development. Most of these studies utilized blood samples collected from cardiac puncture, femoral, ventral abdominal or lingual vein [Whitaker and Wright, 2001] to examine hormone levels [Licht et al., 1983; Zerani et al., 1991] or changes in hematology [Davis et al., 2008]. Due to the potentially invasive nature of the sample collection, these studies were often limited in terms of sample size, methodology and number of species studied. The method of capture and restraint for blood sample collection itself can also induce almost immediate changes in stress and reproductive hormones creating confounding effects [Sapolsky et al., 2000]. However, methods of non-invasive collection of biological samples to measure glucocorticoids can be performed that can serve as a useful tool in studying physiology issues. These non-invasive methods can be used to monitor population differences or individual changes in stress physiology [Sapolsky et al., 2000] resulting from environmental change, such as those that are a result of global climate change [Pounds et al., 2006], changes in habitat and water quality or pH [Alford and Richards, 1999; Blaustein et al., 2010; Blaustein et al., 2012], and the presence of foreign materials such as chemical toxins such as pesticides, herbicides, and fungicides [Bernanke and Köhler, 2009; Köhler and Triebskom, 2013]. The resulting changes in stress hormones can leave populations more susceptible to disease [Warne et al., 2011; Rohr et al., 2013] and potentially alter or inhibit reproduction [Moore and Zoeller, 1985; Paolucci et al., 1990].

Multiple endocrinological questions have already been studied in amphibians using plasma corticosterone, including changes with seasonality [Licht et al., 1983; Zerani et al., 1991] and effects of ex situ environments and increased stress levels [Zerani et al., 1991; Coddington and Cree, 1995]. Stress effects on reproduction have also been studied, with findings that increased levels of stress hormones can be socially important, and even beneficial, leading to increased energy and mobilization needed with seasonal reproduction [Moore and Jessop, 2003]. However, research has found that increased stress for extended periods of time can decrease luteinizing hormone-releasing hormone (LHRH) and testosterone (T5)[Moore and Zoeller, 1985; Paolucci et al., 1990].

Recently, a new method examining and comparing cortisol and corticosterone metabolites in urine to study stress responses in amphibians has been developed and validated in Fijian ground frogs (*Platymantis vitianus*), examining differences between individuals in the wild verses those in a conservation breeding program using an adrenocorticotropic hormone (ACTH) challenge [Sigma A-0298, 0.446 μg ACTH/g bodyweight; Narayan et al., 2010]. This study was able to account for sex differences, seasonality, and post-capture changes in corticosterone. The minimally-invasive nature of urine collection for monitoring stress and reproductive hormones is advantageous in that urine samples are easy to collect and can be stored frozen indefinitely [Monfort, 2003; Narayan, 2014]. Although a physiological stress response can be measured in circulation almost immediately after the event [<1 min, Sapolsky et al., 2000] there is a delay in metabolism of the stimulated glucocorticoids such that the elevated response is often not observed in urine or feces for hours to days after the stimulatory event

[Monfort, 2003]. Once the lag time in the elimination of glucocorticoid metabolites in response to ACTH challenge is determined in a study species, the information can be used to determine the best timing for collection of urine samples for measurement of baseline or maximal stress responses as well as repeated samples within individuals as needed [Narayan et al., 2010].

Similar studies can now be conducted with commercially available corticosterone enzyme immunoassay kits available from companies such as Arbor Assays (e.g., DetectX® K014-H1, www.arborassays.com). However, care must be taken to perform proper biological, physiological, chemical and sometimes comparison assay (antisera) validations [Jiménez et al., 2011], as well as methodological considerations such as sample collection, processing and storage effects for each antisera tested to properly account for potential individual and species specific differences in glucocorticoid and reproductive steroid metabolism [Buchanan and Goldsmith, 2004; Sherrif et al., 2011; Goymann, 2012; Narayan, 2013]. In combination with ACTH challenge, gas chromatographic techniques have been found to be invaluable in determining the appropriateness of the antisera chosen for immunoassay of species-specific and often sex-specific differences [Dehnhard et al., 2003; Touma and Palme, 2005; Lepschy et al., 2007; Shutt et al., 2012]. In ex situ populations, corticosterone has been used to examine effects of capture, ex situ environments, transport, and changes in husbandry methods [Narayan, 2013] or diet [Goymann, 2012]. Future studies should be developed to understand individual variability, effects of stress on reproduction, and effects of stress on immune function and responsiveness. A few questions remain in monitoring stress hormones in amphibians, such as: Which is the best sampling method to use for monitoring stress in amphibians? Can hormones regularly be obtained from other sources such as feces or urine, rather than relying on blood sampling alone?

Instituting a formalized inter-disciplinary research approach has proven to be beneficial not only to the management of current ex situ populations, but also in moving forward with future conservation and reintroductions projects.

CONCLUSIONS

Common themes are found throughout each priority area for amphibian ex situ management. One of the major conclusions is that there is a critical need to design and implement standardized approaches that will facilitate the assessment and evaluation of factors impacting amphibian health. Historically, efforts conducted by colleagues in different fields and institutions worldwide have resulted in a range of different approaches without necessarily having well-defined standards, techniques, tools, measurement units, and protocols in place to evaluate different aspects related to amphibian health. This lack of standardization and resulting significant variability on approaches currently used represents a significant challenge when attempting to objectively and comprehensively evaluate parameters associated with the health and nutritional status of different amphibian species.

When attempting to better understand factors affecting amphibian health, it is important to be able to accurately measure and quantify physiological parameters, disease status, and risk factors associated with individual and population health. Four general areas of improvement were identified in relation to how measurements are currently taken to evaluate ex-situ amphibian health: Infectious Diseases, Husbandry, Genetics and Stress, and Nutrition. To address this challenge, four priorities were identified to better understand different factors impacting amphibian health. A consensus was reached regarding the need to gain knowledge for different amphibian species and life stages. These include:

1. Identify and quantify major health issues affecting ex situ amphibian populations.
2. Identify and coordinate laboratories to conduct analyses using standardized and well-recognized/validated protocols to measure nutritional, infectious diseases, genetic and hormonal parameters.
3. Determine in situ (in the wild in their natural environment) baseline distribution of parameters (e.g., serum nutrients, mortality and fecundity, feeding and reproductive behavior) related to amphibian health.
4. Establish an inter-disciplinary or epidemiological research approach to target specific hypotheses related to amphibian health such as the effects of population genetics (e.g., relatedness, inbreeding) on disease susceptibility, how environmental parameters are related to chronic stress and hormone production, and how immune function differs based on stress.

These four priorities are expanded upon in the summary paper of this issue, which provides recommendations for action necessary to address questions about amphibian health in ex situ populations [Olea-Popelka et al., 2014; this issue].

This overview of nutrition, health, husbandry, and general biology and research aspects of ex situ amphibian care should serve as a foundation for much needed future research in these areas. Despite the multi-faceted approach to ex situ amphibian care, it is evident that population managers must develop more standardized approaches, yet tailor their methods to individual species' needs in order to have successful CBPs for amphibians.

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