ASSOCIATION OF ZOOS & AQUARIUMS

# Amphibian Husbandry Resource Guide

Edited by:
Vicky A. Poole, National Aquarium – Baltimore
Shelly Grow, Association of Zoos & Aquariums
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http://www.aza.org/ConScience/Amphibians_Intro/

ATAG
The Amphibian Taxon Advisory Group

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## Foreword

The Association of Zoos and Aquarium (AZA) Amphibian Taxon Advisory Group (ATAG) created the first version of the *Amphibian Husbandry Resource Guide* in response to the global amphibian crisis as a user-friendly source to aid in the development of successful amphibian conservation programs. As the zoological community continues to employ resources and expand amphibian capacity, *ex situ* management of amphibians remains a crucial component to aid species whose threats in the wild cannot be alleviated in time to halt their extinction. With over 6,900 species of amphibians in the world, there is still much to be learned about their natural history and captive husbandry requirements.[1] This lack of information and expertise can impede the urgent action needed for the 500+ threatened species in risk of disappearing within the immediate future. The zoological community and private sector have made great strides within the last two decades regarding amphibian husbandry and reproduction techniques, and we continue to develop new and innovative methods each year. However, as amphibian populations wane, we must quickly and effectively pool our resources, share our expertise, and learn from our experiences to effectively remain ahead of the extinction tide. Hopefully this second edition of the *Amphibian Husbandry Resource Guide* will not only serve as a resource for amphibian husbandry and management, but will help others solve challenges and create additional space for species in need of immediate conservation.

In addition to this resource guide, the ATAG has produced numerous materials over the past few years to help develop successful amphibian conservation and/or research programs (either *in situ* or *ex situ*; internationally or domestically). These publications include the *Action Plan for* Ex Situ *Amphibian Conservation in the AZA Community* (2007), a detailed description of current amphibian collections and spaces within the AZA community; the *Conservation Resource Manual* (2007) to aid in the development of successful amphibian conservation programs that fit into institution's collection plans, which are appropriate for different levels of resources, and provides species specific action plans and husbandry manuals; the *ATAG Regional Collection Plan* (2008) to guide AZA institutions in collection planning, species management, research and educational outreach; and *Taxon Management Plans for North American and Caribbean* species that have been identified as priority species for conservation action. The AZA has also published *Amphibian Conservation: 2010 Highlights and Accomplishments*, which provides excellent examples of *in situ* and *ex situ* amphibian programs/techniques which could be applied to new programs in the future. All of these resources can be accessed at: www.aza.org/amphibian-population-planning or www.saveamphibians.org. In addition, the ATAG recommends the AZA Professional Development Committee's *Amphibian Biology, Conservation, and Management* course (www.aza.org/prodev/) to improve amphibian husbandry techniques and to benefit from interacting with other amphibian herpetologists, as well as participate in networking opportunities at the annual ATAG meetings.

The contributors to the above-mentioned resources are talented individuals who are always willing to share their expertise and dedicate time and resources to the world in which we happily share with amphibians. For their generosity, I thank them.

The ATAG is here to help. Please feel free to contact me, Diane Barber, ATAG Chair, at dbarber@fortworthzoo.org, or (817) 759-7180 for any question or challenge, large or small.

Sincerely,
Diane Barber

1 The taxonomy of amphibians is always changing; however, the ATAG uses the taxonomy presented by the *Amphibian Species of the World* website (http://research.amnh.org/vz/herpetology/amphibia/).

*Chapter 1*

# General Amphibian Husbandry

Jennifer B. Pramuk[1] and Ron Gagliardo[2]

[1] *Woodland Park Zoo*
*601 N. 59th Street*
*Seattle, WA 98103*
*jennifer.pramuk@zoo.org*

[2] *Amphibian Ark*
*ron@amphibianark.org*

A "red eft" phase eastern newt *(Notopthalmus viridescens)*
(photo courtesy of Brad Wilson, DVM)

## INTRODUCTION

There are many reasons to keep amphibians in captivity including for purposes of exhibition, education, conservation, preservation, and for hobby and personal interests. Historically, zoos have included amphibians within their herpetology programs and displays; however, as they become more conservation-oriented (versus the menageries of the past), zoos will have to alter their collections to reflect their resources and capacities to carry out this work (Rabb, 2004). The financial and spatial requirements necessary to meet conservation goals and propagate critically endangered amphibians are significantly less than those required for larger species (e.g., elephants); be prepared to commit sufficient resources and plan properly for long-term success. The Amphibian Ark (www.AmphibanArk.org) has estimated that approximately 500 species of amphibians are in need of carefully managed *ex situ* help; yet, today likely fewer than 31 species are in managed programs (K. Zippel, pers. comm.).

Amphibians comprise a group of vertebrates that display an enormous diversity of natural histories. Within the three orders, anurans (frogs and toads), salamanders, and caecilians, there are more than 6,900 species (www.amphibiaweb.org) with potentially many hundreds more awaiting discovery and description. To give the reader an idea of how many amphibians remain to be described by science, approximately one quarter of all known amphibian diversity has been described in the past 20 years, with the rate of species discovery not yet having reached a plateau. Within the class Amphibia, lifestyles run the gamut from terrestrial to fully aquatic as adults, with some species even adapting and thriving in arid regions of the world. Reproductive modes range from the "typical" amphibian that is terrestrial as an adult but lays aquatic eggs that hatch into aquatic larvae, to species that brood their eggs within their vocal slits or special pouches on their backs, to females that are viviparous (give live birth). Within vertebrates, only fishes rival this wide range of reproductive modes. Because the ecological characteristics and husbandry requirements of amphibians are so diverse, it is impossible to cover specific guidelines for all groups in this document.

This short guide provides very basic information on how to maintain captive amphibians. Good husbandry practices can circumvent many of the health problems encountered in amphibian collections. Where possible, materials and suggested suppliers are listed and in some cases, alternatives are offered for items that may not be available in all areas. At the end of the chapter, an extensive list of Additional Recommended Literature is provided for those who want to fortify their knowledge of amphibian natural history and husbandry techniques. It is recommended to communicate with others who have worked with that species (or closely related species or genera) in captivity and employ their proven techniques and avoid repeating less fruitful methods. If husbandry experience is unavailable for the target species, methods may have to be tested through trial and error and shared with peers.

Twenty years ago, relatively little was known about amphibian captive care. More recently, a sort of "renaissance" has occurred in the science of amphibian husbandry and breeding. Yet this area of study is still lagging behind the disciplines of mammalian and avian husbandry especially in the areas of nutrition and veterinary care. It is up to you, the next generation of amphibian scientists to fill in our knowledge gaps and improve a field that is still relatively new. To learn more, the Association of Zoos and Aquarium's (AZA) Professional Training Program: *Amphibian Biology, Conservation, and Management* would be an invaluable experience (www.aza.org/ABCM.aspx). The monograph published by this course is a very useful learning tool and many of the topics covered herein are covered there in greater detail. Finally, AZA husbandry manuals for mountain yellow-legged frogs (*Rana muscosa*), Panamanian golden frogs (*Atelopus zeteki*), and Puerto Rican crested toads (*Peltophryne lemur*) recently have been updated and others will soon follow. These manuals are great starting points for any of these species and also provide information applicable to other amphibian propagation programs. The peer-reviewed journal *Herpetological Review*, published by the Society for the Study of Amphibians and Reptiles (SSAR), is a very useful

resource for herpetological husbandry and related techniques. This society provides discounted memberships to zookeepers and students (www.ssarherps.org).

**Planning**

It is important to consider the overall purpose and long-term goals of keeping a particular species in captivity. Goals can range from maintaining amphibians for educational exhibits to reintroduction programs. In addition, it is imperative to gather as much information about the natural history and environmental parameters of the species of choice before proceeding to acquire animals. Extrapolating from related taxa can be useful in some instances where there exists absolutely no precedent for keeping a species in captivity. Select the most closely related species that is closest geographically to the amphibian of interest. Also, especially when little is known about the species or relatives, research the husbandry parameters of a species that shares a similar natural history to the target taxon. For example, if interested in a tropical frog that lives in the leaf litter and lays its eggs on the forest floor, as a starting point consider duplicating husbandry parameters required for a better-known leaf-litter frog such as dart frogs (Dendrobatidae) which are well described in amphibian husbandry literature. The latest and most thorough references on evolutionary history of Amphibia are those of Frost et al. (2006) and Pyron and Wiens (2011). Current taxonomies are presented on two regularly-maintained websites: www.research.amnh.org/vz/herpetology/amphibia/ and www.amphibiaweb.org. Although the published taxonomies are not without their controversies and in some cases are in conflict, often there is something available in the literature on the captive husbandry of at least one species within any given family. Amphibian Ark offers useful information on specific amphibian species in need of conservation action, projects that need collaborators, and practical web-based tools for planning *ex situ* programs on their website (www.amphibianark.org). Especially useful on this site are the Program Implementation and Population Management tools.

At the end of this chapter we provide an extensive list of in-print references, reliable web resources, and links to products that have been used with success by the authors. Note that the listing of products and suppliers does not imply an AZA or ATAG endorsement.

**Acquiring Amphibians**

Obtain amphibians from reliable sources, preferably from captive bred stock or animals that have been harvested in a responsible and sustainable manner. Avoid wild caught amphibians from the pet trade if at all possible but if it is necessary to obtain them from the trade, always request import documentation from the supplier and inquire about the status of the animals being obtained (e.g., how long has the animal been in captivity, what sort of medical treatments have been administered, etc.). Only collect or receive animals from the wild with approval and documentation from the proper authorities. Many states and most countries now require permits for amphibian collection. Moreover, while in the field, one should take measures to prevent the spread of *Batrachochytrium dendrobatidis* (*Bd,* the amphibian chytrid fungus) and other potential pathogens such as ranavirus from one area to the next or from one individual to another. In the field, non-powdered latex or vinyl gloves should be worn at all times when handling adult animals and changed between specimens (if handling tadpoles or larvae, use washed, powder-free vinyl gloves only). Limit handling of animals as much as possible. Keep in mind that harmful pathogens or toxic skin secretions could be transferred easily from one animal to the next. Boots, walking sticks, and other field equipment should be cleaned free of all soil and other debris and if possible, sanitized with household bleach (3–6% sodium hypochlorite) to a 10% dilution for 15 minutes between field sites. This concentration should kill *Bd* along with ranaviruses. Additional fieldwork hygiene protocols can be found in Speare et al. (2004), Zippel et al. (2006), and Pessier and Mendelson (2010).

## Transporting Animals

Transporting amphibians from one location to another should be done with care. Prior to shipping, become familiar with the International Air Transport Association (IATA) standards for transportation of live animals by air.[1] Specimens should be housed in plastic, disposable deli cups or similar containers with tight-fitting lids. The container should be of a size to hold the animal comfortably, but injury may result if the specimen is allowed large amounts of space to jump. Small holes should be cut into lids to allow for gas exchange. Make sure that the holes are sanded down on the inside of the container or punctured from the inside outwards and do not have sharp edges, as amphibian skin abrades easily. The bottoms of the containers should be lined with moist paper towels and/or well-rinsed, moist sphagnum moss. Animals need to be kept cool (generally 65–75 F/ 18–24 C, depending on the species) during transport, by utilizing insulated packaging such as Styrofoam. First, place animals inside plastic containers inside a small cardboard box and then place this box within a larger insulated box. Spaces between plastic containers should be filled with crumpled newspaper to buffer against excessive movement. In extremely warm ambient conditions, sealable plastic lunch bags filled with ice or cool gel packs can protect against overheating. Wrap any temperature packs in newspaper and place within the outer insulated box, but never directly adjacent to containers containing animals. Animals should be shipped the fastest way possible regardless of expense.[2] Slower, more "cost effective" modes of shipment could mean death to the animals, especially during warmer seasons. In temperate climates, it is best to ship animals during the spring or fall, instead of during extreme hot or cold seasons. Although there is a lack of sufficient data to say with certainty how many amphibians die during international transport (Smart and Bride, 1993), some shipments of amphibians have resulted in high mortality due to improper packing (crushing) of animals, overcrowding, overheating, and lack of access to water (Brookland et al., 1985).

## Basic Needs

There are several basic but critically important aspects to keeping amphibians in captivity: 1) enclosures; 2) water (sources and quality); 3) environmental conditions (light, temperature, and humidity); 4) food; 5) natural history and behavior; and 6) veterinary care. Although there are many other nuances of amphibian husbandry to consider, mastering these key parameters for amphibian health, will lay the foundation for maintaining and propagating vigorous animals. This is a general husbandry guide and is merely intended as a starting point for those interested in beginning an amphibian program. Sections addressing breeding methods, larval husbandry, and basic veterinary care are provided at the end. The majority of the world's amphibian species have never been kept, much less bred, in captivity, so there is still much to learn (Pough, 2007). Keeping new species successfully can take months or even years of careful fine tuning to work out proper husbandry protocols. One should not get discouraged by setbacks. Share the development of any discoveries and new techniques with amphibian-minded colleagues. Consider publishing these findings, or else start a blog, join a listserv, whatever it takes to get the word out!

## ENCLOSURES

While some amphibian species might survive in a minimal enclosure such as a plastic box with paper towel and a hide box, it is important to consider other aspects of the health and well-being that can be addressed through proper housing, substrate, and refugia. These aspects, combined with light, temperature, and humidity can directly contribute to the animals' ability to thrive in captivity. Enclosures are best planned and built based on the needs of the species, which are directly tied to its natural history, not necessarily its taxonomy. Consequently, there may be enclosures for terrestrial species such as *Dendrobates, Mantella,* and *Megophrys* (poison frogs and leaf frogs) that are very different from those for arboreal species such as

1 Purchased from the following: http://www.iata.org/ps/publications/Pages/live-animals.aspx.
2 Explore air freight or express shipping (same-day or over-night) options.

*Gastrotheca* or *Rhacophorus* (marsupial and gliding frogs, respectively). Enclosures for riparian species such as harlequin frogs or fully aquatic species such as *Telmatobius* are more examples. Preparatory research on natural history for individual species and applying this knowledge to husbandry cannot be stressed enough.

Regardless of the size, material, or brand, all enclosures should:

- Satisfy the physiological and behavioral needs of the animals
- Prevent escape of specimens and food items
- Be easy to maintain
- Make it easy to monitor the animals

**Construction of Amphibian Enclosures – Materials and Methods**

Commonly-used amphibian enclosures are constructed of glass, acrylic, fiberglass, or other synthetic materials. Using non-porous, easily cleaned materials is important. All enclosures should be fitted with a tight-fitting (e.g., screen) lid but keep ventilation in mind. Some projects, such as head starting programs for ranids, may require larger (e.g., 250 gallon or larger) enclosures (Figure 1). However, for most indoor projects, the most commonly used enclosures are over-the-counter glass aquariums fitted with screen lids (Figure 2). Recently, some companies have begun

**Figure 1.** The head starting program for Oregon spotted frogs (*Rana pretiosa*) at the Woodland Park Zoo uses large Rubbermaid tanks for developing embryos, eggs, and froglets. (Photo: J. Pramuk)

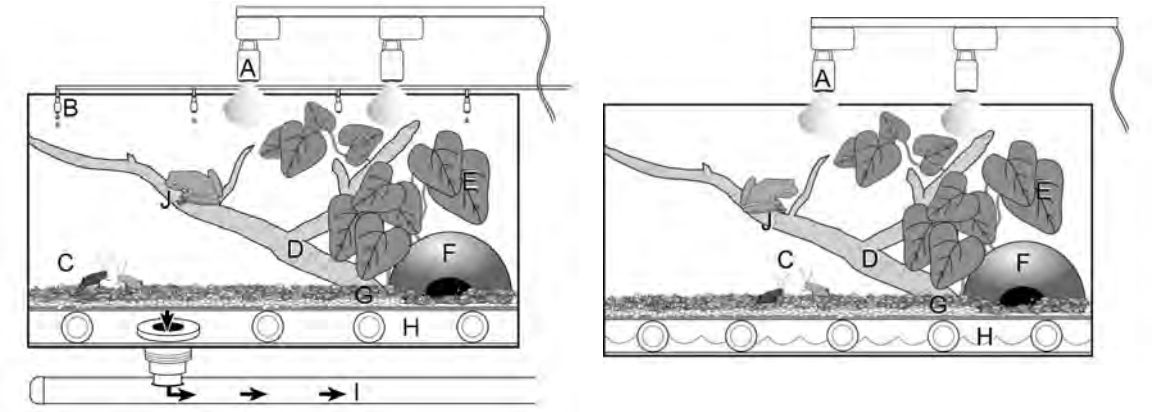



**Figure 2.** Schematics of *open system* (left) and *closed system* (right) terraria with tight-fitting, ventilated lids. **A)** Lighting source (e.g., track lights) to maintain plant growth and UVB for amphibians. **B)** Mist heads, attached to a pump, which deliver aged or filtered water to the terrarium (operated manually or via a timer; open system only). **C)** Invertebrate food (e.g., vitamin-dusted and gut loaded domestic crickets) adds ammonia to the terrarium from their waste and dead bodies. **D)** Cage furnishings (e.g., a cut branch from a non-toxic plant). **E)** Live foliage, which consumes some ammonia and other nitrogenous wastes from the substrate. **F)** A hiding spot for terrestrial amphibians (e.g., an inverted coconut shell with a door cut into its side). **G)** A substrate layer (e.g., pea-sized gravel covered with moist sphagnum moss and leaf litter). **H)** A false bottom, which can be constructed from egg crate or perforated PVC sheeting, sitting atop small supports (e.g., cut pieces of PVC pipe). The false bottom is covered with a sheet of fiberglass window screen to prevent substrate, invertebrates, and animals from reaching the drain and escaping. Note the wastewater reservoir under the false bottom in the closed system. **I)** The drain, cut through a hole in the bottom of the terrarium and fitted with a bulkhead plumbed to a PVC pipe, which allows excess water and waste to be removed on a continual basis from the open system. **J)** The terrarium's resident frog. (Illustration: J. Pramuk)

manufacturing amphibian-specific terraria[3]; despite their increased cost (ranging from $40–300, initial reports are that they are worth the extra expense for ease of cleaning and access.

An alternative to glass aquaria are polycarbonate food grade storage containers.[4] The lids of these containers can be modified to provide sufficient air exchange by cutting out a portion and gluing in fiberglass window screen with a hot glue gun or silicone aquarium sealant. Although not as aesthetically pleasing as glass enclosures, these serve well as off-exhibit housing. Acrylic or polycarbonate enclosures can be expensive initially, however they are lighter than glass and more resistant to cracking and breaking; in the long run, they are probably more cost effective. The downside of plastic enclosures is that they scratch more readily and require frequent buffing to keep clear. One salamander breeder in the U.S. uses these simple enclosures for an entire colony with great success in maintenance and breeding.

For many terrestrial salamanders and smaller terrestrial frogs, 20 gallon Long aquariums with tight fitting lids work well. Arboreal, nervous, and/or jumpy species seem very prone to escape and injury from many enclosures. A method employing a ten-gallon aquarium set on end and outfitted with a front opening door and ventilation area serves well for housing glass frogs (centrolenids) and colonies of juvenile phyllomedusines (Figure 3). Add tree branches and plants to offer sufficient climbing and perching areas.

**Figure 3.** Vertical tanks used for housing arboreal frogs. Note the simplistic set-up including a floor with wet paper towels instead of gravel or soil, which can harbor parasites. (Photo: R. Gagliardo)

**To Plumb or Not to Plumb?**

It is important to recognize that any enclosure, regardless of size, shape, or use, represents a living ecosystem whereby resources (animals, water, light, heat, etc.) are added and that as an ecosystem, natural biological processes should be allowed to occur. There are two fundamental types of systems with regards to amphibian enclosures: *closed* and *open* (Figure 2). Closed systems do not allow the outflow of water and wastes and thus require close attention to drainage and hygiene. Open systems typically have built-in ways of dispensing of excess waste products through drains, overflows, and the ability to rinse the enclosure easily and frequently on a regular basis. Open systems require more complicated plumbing to allow fresh water in and wastes to exit the system, but the advantages for hygiene are often well worth the extra effort.

The decision to build and maintain freestanding (i.e., closed) enclosures or plumbed (i.e., open) terraria will depend on the needs of the project. If the species or program requires highly hygienic conditions, plumbed enclosures are likely to be preferable. This type of terrarium will require greater initial monetary and time investment to set up, but over the long haul, will save time because they are faster to clean. Regardless of if the tank is plumbed or freestanding, several guidelines apply to set up both types of enclosure.

3 ExoTerra® and ZooMed® both sell quality terraria specifically for amphibians.
4 Such as Rubbermaid® or Sterlite®

**Setting Up an Enclosure**

It is recommended to use a clean substrate, such as gravel or lightweight expanded clay aggregate (LECA)[5], from a reliable source for the bottom of the tank. The substrate provides the basis for a healthy tank ecosystem by creating humidity and harboring beneficial bacteria and small invertebrates that help break down waste. Make a three or four inch (7.5–10 cm) square depression in the gravel or other substrate in one corner of the aquarium's floor or grade the substrate to form a small pool at the shallow end for the animals to access. The gravel layer should be completely covered with at least a one inch (2 cm) thick layer of sphagnum moss or sheet moss. The moss should be soaked overnight and rinsed thoroughly prior to use in an enclosure. Ideally, this sort of environment should be set up at least two weeks prior to introducing animals so that the plants can become established and beneficial bacteria (i.e., natural, biological filtration) will be ready to breakdown wastes. Cork bark and plants can be added to the background, but it is recommended that all or most soil is removed from the roots, as it can harbor potentially harmful parasites such as nematodes. Many bare root plants (e.g., pothos, *Spathiphyllum*, etc.) will do well when planted in a semi-aquatic environment.

The water level in the gravel should be maintained at least 0.6 inch (1.5 cm) below the upper level of the substrate (i.e., sphagnum or sheet moss). Through capillary action, the water will slowly wick its way up through the substrate, offering moist conditions for the plants while the rest evaporates, providing sufficient humidity. Coconut (coco) fiber can also be used as a substrate on top of the gravel and is long-lasting, holds moisture, and is free of unwanted organisms such as worms or flagellates. Because coco fiber is friable, a substrate divider should be used to keep this layer from mixing with the gravel or LECA layer below. Fiberglass window screen or shade-cloth cut to fit the footprint of the tank works well as a divider for the two layers. Live java or cushion moss, or hydrated and rinsed sphagnum moss can be used on top of the coco fiber. Sphagnum moss has an advantage over sheet moss in that the former will usually develop live growth when maintained under high humidity and moderate light.

An enclosure outfitted with a false bottom reduces the weight of the tank by allowing the use of less gravel and improves flow through the substrate if a drain is used (i.e., open system). Alternatively, a similar thickness of LECA substrate (very popular with dart frog culturists) provides a well-drained lower substrate. LECA is slightly more expensive than aquarium gravel, but it is much lighter and can be used in situations where weight is an issue. To raise and separate the amphibians and substrate from the wastewater and drain, create a false bottom using egg crate[6] (i.e., sheets of plastic light diffusion grid commonly used with fluorescent lights) or perforated PVC by following the instructions in the box.

**How to Create a False Bottom**

- Cut a piece of egg crate or a sheet of perforated PVC to the shape of the enclosure bottom, forming a false bottom.
- Cover the false bottom in fiberglass window screen.
- Tack the screen onto the false bottom with dabs of silicone or with a soldering iron.
- Scatter several one to two inch (2–5 cm) pieces of PVC on the bottom of the enclosure to support a false bottom.
- Rest the false bottom on top of the PVC pieces.

The false bottom is now ready to support a substrate layer.

Plant roots need oxygen and too much water can kill them, so it is important to allow excess water to drain in order to keep the substrate from becoming saturated and anaerobic. Using a

5 Such as Terra Lite®
6 Available at most hardware or lighting specialty stores

drainage substrate such as gravel or LECA is helpful in allowing excess water and waste to flow out of the substrate (see section on Substrates for more information).

**Rain Chambers**
Many amphibians breed during the rainy season, which can easily be duplicated under controlled conditions *ex situ,* through the use of a *rain chamber.* However, relocating specimens from their living enclosures into a dedicated breeding enclosure (i.e., a rain chamber) may disrupt the breeding cycle. It is prudent to move either the animals well-prior to initiating pre-breeding conditioning (weeks to months in advance), or else to utilize the specimen's current living enclosure and installing the rain chamber there.

There are a variety of ways to set up a rain chamber. The most common method is to use a tank outfitted with a false bottom, drain, and a sump container with a submersible pump which returns water to a spray bar made from a length of PVC pipe (Figure 4). The PVC spray bar can be modified with different numbers, positions, and sizes of holes to achieve various degrees of water coverage and force. The pump is plugged into a timer so that rain cycles can be regulated. Rain chambers may be fashioned from tall aquariums with false bottoms, large plastic trashcans, and even five gallon buckets. If submersible pumps are not available, canister filters may be used to run mist bars. Rain chambers can also be set up using tanks that drain to building wastewater lines (an open system).

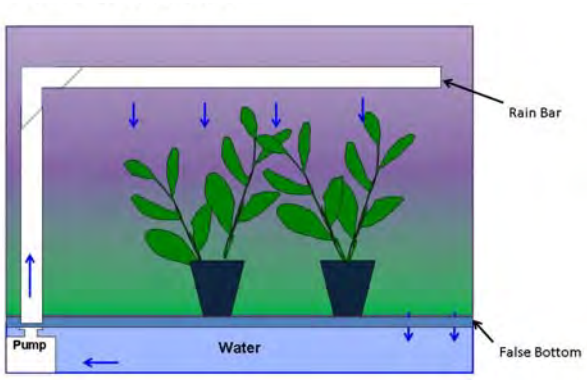



**Figure 4.** General rain chamber configuration. (Credit: R. Gagliardo)

**Cleaning Enclosures**
When breaking down and cleaning enclosures they should be rinsed with water and can be disinfected with household bleach (3–6% sodium hypochlorite) to a 10% dilution. If bleach is used, rinse tanks thoroughly and allow to air dry completely, or use sodium thiosulfate to neutralize the bleach.

**Freestanding Vivaria (i.e., closed systems)**
Advantages of freestanding vivaria are that they are easy and quick to assemble, are not attached to a complicated plumbing system (and thus can be moved more easily), and can be more easily isolated (for cleaning, disease management, etc). Remember that enclosures without drains must maintain a careful balance of beneficial bacterial and fungi to break down wastes.

Proper removal of waste is particularly important for keeping a closed vivarium healthy for reducing levels of harmful bacteria and waste products. When applicable, the use of live plants to absorb nitrogenous wastes is an excellent way to maintain a healthy ecosystem in an enclosure without a drain. Be aware that in closed systems excess wastewater accumulates; more diligence is required to prevent waste products from reaching toxic levels in smaller enclosures. An appropriate, basic closed system vivarium can be created easily by adding approximately two inches of washed, pea-sized aquarium gravel to a glass aquarium. Another simple type of closed system is a shoe box with paper towel substrate.

**Plumbed Vivaria (i.e., open systems)**
Allowing water and dissolved wastes to leave the system via drains is an excellent method to maintain good hygiene, whereas closed systems run the risks of accumulating toxins and

waste products. Additionally, plumbed vivaria are most suitable in situations where moisture is being added constantly (e.g., rain chamber or automatic mist cycles). These systems can be used for both display tanks and more utilitarian off-exhibit set ups. To accommodate a drain in a glass tank, a hole should be drilled into the bottom with a diamond-tipped drill bit [approximately 1–1.5 inch (2–4 cm)], using a powerful drill (e.g., 14–18 volt). Be sure to wear proper eye protection and leather gloves when working with power tools and glass. While drilling, run a stream of tepid water over the area being drilled to reduce heat buildup which can crack the glass; if working in an area that cannot be flooded with a hose, use a dam made of duct tape, clay, or putty around the hole to be drilled to create a small reservoir of water. Maintain the drill at a 90˚ angle to the glass surface, pressing slowly and firmly to avoid cracking the glass (Figure 5). Once completed, the hole can be fitted with an appropriate sized plastic bulkhead siliconed into place to be used for a drain.[7] The bulkhead serves as a connector to the external drain pipe and also protects people and animals from the sharp edges of the hole.

**Figure 5.** Demonstration of how to drill a hole in a glass aquarium using a diamond-tipped drill bit. Photographed at the *AZA Amphibian Biology, Conservation, and Management* course where everyone gets to try their hand at drilling a tank. (Photo: J. Pramuk)

Affix a plastic stopcock at the base of the PVC pipe leading from the tank drain to a central drain. The stopcock will allow control of the rate at which water drains from the tank and allows tanks to be flushed regularly with purified or dechlorinated room temperature water. Open systems usually do not require filtration, as the misters are flushing and dumping the water in an enclosure on a constant basis. Most bulkheads are threaded, allowing the installation of a standpipe of any desired height, which can be adjusted seasonally to provide a breeding pool (Figure 6). Pool depths vary depending on the needs of each species. Standpipes should have a screen or similar mesh cover to prevent the escape or accidental flushing of animals or food items, and should be monitored to prevent clogging and possible overflow. More elaborate external standpipes have been used with great success and additional expense (Figure 7).

**Figure 6.** An internal standpipe used to regulate the water level inside a terrarium. (Photo: R. Gagliardo)

**Figure 7.** An external standpipe used to regulate the water level inside a terrarium. (Photo: R. Gagliardo)

7 Bulkheads are available from US Plastics, Aquatic Ecosystems, or McMaster-Carr.

If planning a large collection where hand misting would be too labor intensive or keeping species which require frequent misting or are large-bodied (i.e., produce more waste), an automated or semi-automated misting system should be used, where excess water flows into a drain at the bottom of the enclosure. Frequent flushing ensures that wastes do not accumulate in the enclosure. Automated misters can be set up on timers, misting as often as required for the species in the collection. However, do not allow the incorporation of automatic watering systems to take the place of careful, daily observation. Plumbed vivaria allow the keeper to maintain a high level of cleanliness. Frequent flushing of the system also may reduce parasite (e.g., nematode) loads in the enclosure.

**Figure 8.** A large terrarium set up for highly hygienic conditions. The keeper uses a spray nozzle to flush feces and other debris from the enclosure. Waste products are flushed through a layer of egg crate, covered in fiberglass window screen, down a drain drilled into the floor of the enclosure. (Photo: R. Gagliardo)

**The Hygienic Enclosure**

There is often a decision to be made between housing an animal within a naturalistic enclosure or, at the other extreme, a sterile box with a paper towel. An appropriate compromise may include using a system that incorporates an enclosure fitted with drainage, a false bottom, and potted plants (with all potting soil removed). This type of enclosure has proven useful in preventing the parasite buildup that may occur in either closed or open systems that contain substantial substrates. The simplicity of this set up makes cleaning easy without too much time and effort, also allowing for efficient fecal test monitoring (Figure 8).

**Figure 9.** An open system for quarantine. (Photo: J. Pramuk)

A simple enclosure can be built from plastic storage containers (Figure 9). These work particularly well for quarantine situations when an inexpensive, temporary, and highly hygienic enclosure is required. These containers can be constructed so that effluent empties into plastic buckets (open system). To help prevent transfer of potential pathogens into municipal wastewater, effluent can be sanitized with sodium hypochlorite prior to dumping it down the drain.

Another type of enclosure that functions as an open system utilizes commercially-available screen enclosures[8] or can be constructed using a box frame made from PVC pipe covered with a sewn, plastic mesh bag or sleeve (Figure 10). These

**Figure 10.** Lightweight, screen enclosures outfitted with potted plants and cork bark work well for large, arboreal frogs and are easy to move between various climate-zoned areas. (Photo: R. Gagliardo)

8 Commercially known as *Reptariums,* available from most pet product dealers.

lightweight screened enclosures are easy to assemble, move, and clean via periodic rinsing of the mesh sides and bottom. Waste can be removed by manually removing larger pieces and then rinsing the enclosure with filtered or aged water. This type of enclosure has proven very useful in outdoor applications for phyllomedusine frogs that enjoy basking and for large hylids that would damage themselves by jumping around inside a glass enclosure.

## Components of Amphibian Enclosures

All materials within tanks can be used to offer diverse environments based on a species' natural history and wild habitat.

### Display Backgrounds

*Concrete:* Concrete is sturdy, long-lasting, and can appear naturalistic if dyed to match the color of soil or mud (Figure 11), but there are several drawbacks including its excessive weight and porous nature. Additionally, highly-active frogs tend to abrade their noses against rough concrete while pursuing prey, which can lead to systemic infections and death (J. Pramuk, pers. obs.); during installation, have the fabricator smooth the concrete surface as much as possible before it dries. Concrete leaches lime for a long time after it has cured, creating a dangerously high (basic) pH that may be toxic to amphibians. Acid washes can resolve this problem, as long as the concrete is well cured; however, the pH should be tested periodically for several months before amphibians are introduced. Sealing the concrete will also help with porosity. If inexperienced with acid wash or concrete sealing procedures, consult an experienced contractor for assistance.

**Figure 11.** An amphibian exhibit with a concrete background. This is a closed-system enclosure with a combination of mechanical and biological filtration that produces a waterfall trickling down the back of the exhibit. Waterfalls are aesthetically pleasing and aerate the water. (Photo: J. Pramuk)

*Fiberglass and resins:* The benefits of fiberglass and resin-based backgrounds are that these materials are lightweight and can be shaped easily to resemble rockwork, mud banks, etc. Unfortunately, fiberglass and resin-based compounds will release chemical vapors (*off gas*), which can be toxic to animals or plants. One advantage is that the toxic phase for these substances is shorter than that for concrete and they usually are inert after they cure. Both of these materials are more difficult to work with than concrete and may require specialized training, proper ventilation, and personal protection devices (PPDs), but the results are aesthetically pleasing and long lasting.

### Substrates

When choosing a substrate, first consider the needs of the animals and determine whether the enclosure is to be more aesthetically pleasing or easier to clean. There are many inexpensive substrates (e.g., gravel) as well as more costly amphibian-specific ones available on the market; gravel works well as an inexpensive starting substrate. The benefit of most naturalistic substrates is that they provide burrowing species places to hide. The pH of the substrate also should be considered, for example some mosses are rather acidic which may irritate some species' skin.

*Quarantine or hygienic situations:*

- Sphagnum moss: High-quality sphagnum moss is soft, provides more burrowing/hiding opportunities (refugia), and has antimicrobial properties that can be

a good choice for some quarantine applications. Used moss can also be sterilized and re-purposed for other applications.

- Astroturf: This plastic grass-like material is inert and easy to disinfect, as long as it is rinsed thoroughly afterwards, and can be cut to fit any size or shaped enclosure. It is mold-resistant and can be maintained in semi-aquatic enclosures. Larger, heartier species such as *Rhinella marina* that defecate frequently and abundantly might do best on this substrate.
- Paper towels: Paper towels work well in quarantine situations but may dry out quickly, requiring frequent monitoring to ensure that they are sufficiently moist. The moist environment also provides a substrate perfect for bacterial growth, but daily changes can keep problems to a minimum; it is recommended to change the paper towels at least every 48 hours, if not sooner. Unbleached paper towels are also recommended, as white or bleached paper towels may contain traces of chemicals such as dioxin.
- Pulp fiber pet bedding[9]: This option is reported as a suitable alternative to soil for caecilians and other burrowing amphibians (D. Fenolio, pers. comm.). This substrate should be rehydrated thoroughly with excess water squeezed out prior to use.
- Foam rubber: Foam rubber can be purchased in various thicknesses from fabric stores. This material can be cut to any shape and works well for quarantine situations where cleanliness is a priority, as it can be disinfected. It can also be used for isolated specimens that need or require a softer surface for medical purposes (i.e., broken limbs or rostral rubs). There is some concern about the potential release of dioxins with foam and its potential to harbor harmful bacteria.

*Organic and natural substrates*:

- Coconut (coco) fiber: This substrate has grown in popularity because it is resistant to breakdown, lasts for a year or more, and makes an environmentally friendly alternative to peat moss. It comes dried and compressed into bricks for easy shipping. Soak the coco fiber brick over night in water and squeeze out excess moisture prior to placing it at the bottom of the enclosure. If used on top of a layer of gravel, insert a piece of shade cloth or fiberglass screen cut to fit between the gravel and the fiber to prevent the fiber from mixing with the gravel.
- Moss: Mosses should be soaked for at least 24 hours and rinsed thoroughly before use, producing a soft, moist substrate that is easily changed. In quarantine situations or others where the moss is not used for long, it is possible to heat sterilize the moss and recycle it for horticultural applications. Live mosses can be collected locally, but it is possible for these moist mosses to retain *Bd* that could infect a collection (*Bd* spores are most easily transferred from one moist surface to another). Some culturists treat the live moss with a diluted itraconazole (0.01%) solution prior to use in amphibian enclosures but this may not effectively reach every zoospore in the moss. Considering the risks and consequences to a collection, collecting live moss for the terrarium is not recommended. New Zealand or Chilean sphagnum moss is superior to other types of sphagnum (e.g., Wisconsin sphagnum). Sphagnum mosses tend to have a low pH, which may irritate the skin of certain species.
- Modified orchid substrate: For long-term (3 years +) use in terraria, a modified orchid media developed at the Atlanta Botanical Garden has shown some promise (see sidebar below for recipe). This mixture was developed for growing tropical epiphytes where moist, acidic, and well-drained conditions are needed. With proper drainage below, epiphytes such as bromeliads, aroids, and even some orchids can be grown terrestrially within the amphibian enclosure.

9 Such as Carefresh Pet Bedding®

- Potting soil: In general, soil is not a good choice for use with amphibians as this industry is poorly regulated in terms of components. Potting soil tends to become compacted and become permanently oversaturated with water under terrarium conditions. If there is no other alternative, use only steam-sterilized potting mix without vermiculite, perlite, or other artificial additives such as fertilizers. Potting soil can harbor and encourage the establishment of nematodes and other parasites, so its use should be limited. However, some fossorial amphibians (e.g., spadefoot toads and many salamanders) do best on a soil substrate and using organic soil products and substitutes may be appropriate in these cases.
- Rocks and gravel: Gravel is a useful, inexpensive, and relatively easy to clean substrate. It is widely available from most pet supply dealers and lawn and garden centers, coming in a variety of sizes and colors. However, it is heavy and can lend an unnatural look to the vivarium if the appearance is overly uniform. Be careful that animals, especially aggressive feeders such as horned frogs (*Ceratophrys* spp.), do not ingest the gravel by accident, as this can cause a digestive track impaction. A generous layer of moss on top of the gravel layer can reduce the risk of accidental gravel ingestion.
- Sand: Play sand is relatively inexpensive and usually well rinsed prior to packaging. Select a grain size that is not powdery, but that has some substance to it. Note that sand, if consumed in substantial quantities, can pose an impaction hazard similar to gravel. A calcium-fortified sand[10] has been employed to aestivate species such as Budgett's frogs (*Lepidobatrachus laevis*).

**Modified Orchid Substrate**
(Atlanta Botanical Garden)

1 part peat moss
1 part fine horticultural charcoal
2 parts fine orchid (fir) bark
2 parts milled sphagnum
1 part medium tree fern fiber

Mix and moisten well for 24 hours before use (if possible), as components tend to be very dry!

Ecologically friendly substitutes
- Coco fiber in place of peat moss (see www.peatmoss.com for information on environmental restoration practices from peat moss producers).
- Bruc Fiber (twigs of an ericaceous shrub, Erica arborea, harvested in the Mediterranean region) in place of tree fern fiber.

**Enclosure Furnishings**

Furniture, refugia, and landscaping are important to the well-being of animals. It can be simple (i.e., a wet paper towel as substrate and an inverted dish with a door cut into it as a hut), or as naturalistic as desired. Regardless, all amphibians should be offered plenty of hiding spots in their home to provide sanctuary. With the exception of relatively few usually toxic diurnal species, in the wild most amphibians are nocturnal and considered prey by just about every other carnivore in their habitat. Providing a few accoutrements to give them some security will pay off with more stress-free, healthier animals. Cork bark; dried leaves; coconut huts (coconut halves turned upside down with a little door cut into it); inverted, opaque plastic tubs with an opening cut into one side; scrap PVC pieces; and film canisters can all make great hiding places (Figure 12). Cork tubes work well as refugia for species that dwell and breed in

**Figure 12.** *Mantella pulchra* using a corkbark hiding spot. The dish on the left is an insect feeding station, which prevents insects from dispersing too rapidly and allows frogs to take their time feeding. A short piece of PVC on the lower right offers an alternative refuge. (Photo: J. Pramuk)

10 Such as Calisand®, Vitasand®, Repilite®

tree holes. For burrowing species (e.g., gopher frogs), PVC tubes can be buried partially in the substrate to simulate a burrow.

Refugia need to be opaque. Heavy or unstable objects should be avoided, as they could fall and crush animals. Some herpetoculturists obscure one outside wall of the enclosure to increase seclusion and provide visual barriers between tanks. Avoid black covers which makes glass more reflective on the inside, encouraging frogs to jump against the wall, potentially causing cranial trauma. Attractive false rock walls can be made from a wall of cork bark affixed with silicone onto the back of the enclosure. Be cautious of potential gaps where frogs can become wedged between the cork and glass.

A quick and easy method to obscure the inside walls of an enclosure while also providing a substrate for plants utilizes silicone, peat moss or coco peat, and crushed tree fern or bruc fiber. Place enclosure on its side and spread a thin layer of silicone over the entire surface with a wide putty knife. Wearing gloves, sprinkle tree fern or bruc fiber over the surface to desired effect, and carefully press into silicone to insure good adhesion. Next, sprinkle dry coco peat or peat moss liberally over entire surface, filling in spaces among the tree fern or bruc. Pat down and allow the silicone to cure for 12 hours before turning enclosure over to allow excess peat to fall away. This creates an excellent surface for mosses and other creeping plants, such as *Ficus pumila* or *Philodendron* spp. Taking this a step further, customized "rock" walls can be created by laying down a layer of expanding spray foam[11], carving it to resemble rock once dry, and coating it with a layer of black aquarium silicone infused with coco fiber or waterproof epoxy resin. Pockets can be carved into the foam to hold small plants. Silicones for aquatic applications[12] are slightly more expensive than aquarium or household silicone, but are more durable. All silicones used need to be 100% silicone with no potentially toxic additives.

Be sure that everything that goes into an enclosure (both organic and inorganic) is not contaminated with soap, bleach, pesticides, or other chemicals. If items such as gravel, rocks, decorations, or plants are added make sure they too are free of contaminants. Occasionally, rocks and gravel contain toxic residuals from processing. Some plastic plants are not intended for use in water and may contaminate the enclosure. Be careful when collecting natural items from an area where amphibians abound. Moist surfaces (e.g., mosses, soils, wet leaves) can harbor parasites and *Bd* spores that can infect captive animals. Due to potential exposure to *Bd* or ranavirus, if gathering material from the wild, allow them to dry completely or expose them to high heat prior to use. It is suggested that soil or other organic objects be microwaved or steam sterilized prior to placing them into the enclosure. See Pessier and Mendelson (2010) for additional information on disinfection.

Wood pieces are beautiful and naturalistic, providing climbing, hiding, or basking areas for amphibians. Epiphytic (i.e., air) plants or vines can be mounted to wood, adding a naturalistic flair. Many types of readily available wood will work fine in most situations; however, in environments with greater humidity, many woods will rapidly begin to decay. Some of the best choices for wet enclosures include cork, cypress, and mopani wood. Terraces can be created using cork or other wood, which can then be planted with vines, java moss, or other plants. Terraces also partition the space which may be controlled by territorial males of certain species of frogs such as mantellas and dendrobatids. Most terrestrial salamanders require plenty of cover, i.e., bark and wooden pieces, as well as a thick layer of dried leaves, which they use to burrow underneath and hide.

11 Such as Great Stuff Gaps and Cracks®
12 Such as Dow Corning® 795

**Plants**

Artificial (e.g., plastic or "silk") plants can be used for amphibian tanks and work well in situations where hygiene and biosecurity are concerns. An added benefit to artificial plants is that there is a reduced risk of spreading *Bd* or other amphibian diseases. There is some anecdotal evidence that amphibians prefer live plants. In addition, live plants act as biological filters by absorbing nitrogenous wastes effectively in enclosures. Consider the following factors when selecting plants: 1) adaptability to high-humidity conditions; 2) non-toxic to animals; 3) compatibility with the species' natural history; and 4) native to the same region as the animal(s), conveying biological accuracy.

High-humidity plants such as pothos species (e.g., *Scindapsus aureum*), ferns, tropical and temperate ivys, *Selaginella* and other club mosses, java moss; moisture-tolerant bromeliads[13] such as *Guzmania, Vriesia, Neoregelia,* and some *Tillandsia*; creeping or other species of *Ficus*; and aroids of the family Araceae are commonly used in terraria. *Peperomias*, begonias, and calatheas also work well. Orchids are increasing in popularity and their use in the terrarium is becoming more common. Terrestrial jewel orchids are susceptible to rotting in moist environments and may not be the best choice for a beginner. More success has been seen with smaller, epiphytic species mounted on branches and placed in areas where good air-circulation and drainage allows proper drying of root systems. Submerged, aquatic plants should be provided for salamanders, as the eggs of many species are adhered either singly or in clusters to submerged plant leaves. Species of aquatic plants that can be cultured easily include java fern, java moss, and the ubiquitous *Elodea*.

Some species of potted plants purchased at the local greenhouse contain toxins that may be unwittingly consumed by animals when crickets or other prey items ingest the plant material. For example, oxalate-producing plants (e.g., silver queen, *Aglaonema roebelinii*) have been linked to subcutaneous edema and lethargy in waxy frogs (*Phyllomedusa sauvagii*) and feeder crickets were suspected of eating the plants in the terrarium before the frogs consumed the crickets (Wright and Whitaker, 2001). Avoid using other high oxalate-producing plants such as dumb cane (*Dieffenbachia* sp.). There is also some evidence that some plants in the Commeleniaceae (spiderwort) family are toxic (R. Gagliardo, pers. obs.). A little research on appropriate plants will pay off in the long run for animal well-being.

In addition to commercial suppliers, consider liaising with a local botanical garden as a source of plants and propagules. Another excellent source of a variety of material is the plant tissue culture industry. Plant tissue culture plugs or "liners" are excellent, inexpensive sources of small plants that can be grown up on site for future use in amphibian enclosures. Extra, vacant aquariums with lights mounted above make great holding areas for plant material until needed in terraria.

For more hygienic enclosures where appearance is less important, modular potted plants can be very useful. These are prepared using net pots (from hydroponics suppliers), gravel or moss/charcoal substrates and either tissue culture plugs or other propagules. They provide cover, perching and egg deposition sites (Figure 13), and

**Figure 13.** A modular potted plant used in an off-exhibit enclosure. (Photo: R. Gagliardo)

13 Plants of the pineapple family whose water-filled axils are essential for leaf-axil breeders such as *Oophaga* (*Dendrobates*) *pumilio.*

offer the additional advantage for hygienic enclosures in that they are easily removed for cleaning.

**WATER**

Both quantity and quality of water are important considerations and are among the most important factors aiding an amphibian's survival. Unlike reptiles, amphibians do not have a shelled (amniotic) egg. Their relatively unprotected eggs are essentially part of the aquatic environment in which they are bathed and the developing embryo is subjected to whatever water quality problems are present. Amphibians are perhaps even more sensitive to water quality than many fishes; consequently, aquarists, because they are aware of water quality issues, often make the best amphibian caretakers. Amphibians do not drink from their mouths but instead absorb all or most of their moisture through their highly permeable skin (in anurans, water intake is primarily through a highly-vascularized "drink patch", located on the posterior portion of their belly). They also absorb a significant portion of their oxygen through their skin. If their skin desiccates, amphibians will lose the ability to exchange gases through their skin and will effectively suffocate. Unfortunately, an amphibian's amazing adaptations and strong ties to an aquatic environ also mean that they are particularly sensitive to changes in water quality and quantity.

Water temperature, pH, ammonia, and nitrite should be tested daily in new enclosures to ensure that conditions are appropriate for the new amphibian inhabitants. Relatively inexpensive colorimetric water quality chemical test kits can be purchased for less than $20 at most pet stores (Figure 14). These reagents are added to a sample of water, which results in a color change that is compared to a color chart, identifying a specific range of levels in the water. Additionally, more expensive but more accurate spectrophotometers can be used to measure these parameters. Testing results are more accurate, but not necessarily more useful, as colorimetric methods are significantly more cost effective and will usually be sufficient to indicate if water is within normal ranges or not. Newly constructed terraria often will undergo an ammonia spike several days following set-up, and ensure that equilibrium has been maintained prior to introducing amphibians to the enclosure. It is recommended to set up an enclosure several weeks to a month prior to introducing animals. This will allow natural bacteria to become established in the substrate and plant growth to become lush. Both the bacteria and plants will act as biological filtration. After animals are introduced, water quality should be tested periodically and whenever mortalities occur, to troubleshoot the cause of death. Keeping a daily water quality log will provide baseline data and can target problems before they become harmful to the animals.

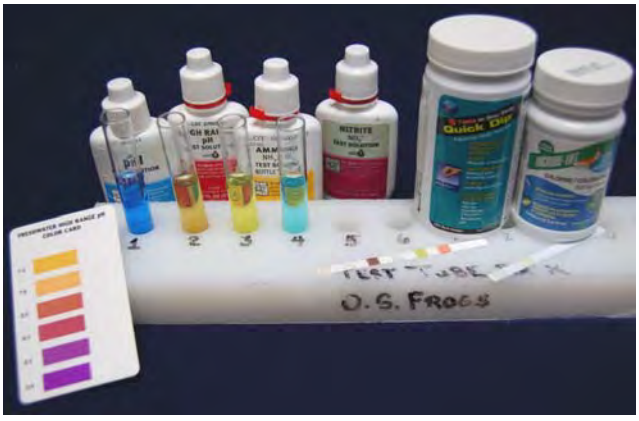



**Figure 14.** Two types of colorimetric water quality testing kits: on the left are freshwater liquid tests that are mixed in a test tube; on the right are two types of paper strip testing kits (5-in-one and chlorine/chloramine). Both tests result in a colored sample that is compared with a chart to reveal the level of pH, or parts per million (ppm) of the chemical being tested. These tests are relatively inexpensive and an easy way to perform quick tests of water quality which are invaluable for troubleshooting and

The importance of water quality cannot be overstated. For more detailed information refer to Odum and Zippel (2008), Whitaker (2001), Browne et al. (2007), or register for the AZA

*Amphibian Biology Conservation, and Management* course, from which much of the below information is summarized (Odum and Zippel, 2004).

**Chlorine and Phosphate**

Chlorine is the most toxic substance to be contended with in source water. Unfortunately, this chemical will be encountered because most municipal water treatment plants use it to kill bacteria in drinking water. Even minute amounts can cause distress or death in fishes and amphibians; sensitivity varies greatly across species and life stages. For example, tadpoles are more vulnerable to chlorine and other water quality issues than adult amphibians because they breathe through their gills which are particularly sensitive to chlorine's effects. It is recommended that all chlorine be removed from water prior to its use on collection animals. Chlorine test kits are commercially available, and source water should be tested on a routine schedule at various times of day, as chlorine concentrations in municipal water supplies fluctuate.

Aging the water (having it to sit for a day or two) prior to use, allows free chlorine ($Cl_2$) to dissipate in the form of gas. The process of dechlorinating can be accelerated through aerating (using an air stone), heating the water, or using an activated carbon pre-filter on source water. However, this will not get rid of chloramines, if they are used in place of chlorine as the antibacterial agent in the municipal water source. In this case, using a water conditioner (e.g., sodium thiosulfate) is recommended if not using a reverse osmosis (RO) or other filtration device. Sodium thiosulfate can be purchased in bulk and made into a supersaturated solution. To make a supersaturated solution, mix crystals with room temperature water until no more will go into solution. The solution can be stored in a chemist's 250 ml transparent squeeze bottles for convenience. Be aware that when thiosulfate reacts with chloramines, toxic ammonia is produced in small amounts, which will need to be handled with appropriate filtration.

Many older cities add phosphates to their municipal water to chelate (bind) lead used in older pipes from the water supply. Unfortunately, excess phosphates are bad for amphibians as they bind calcium. A high phosphorus:calcium ratio can lead to serious neurological and osteological problems, paralysis, and even death. Phosphates usually are too small to be removed via RO, but they can be removed via phosphate sponges[14], arsenic filters (Figure 15), or other chemical filtration methods. If phosphate is a continued problem, consider installing a distillation unit and then reconstituting the product with salts to make isotonic "reconstituted water" (see below).


**Figure 15.** A water filtration assembly for removing chlorine and phosphate . A) Air handling unit; B) Air filter placed over the air intake of the room; C) Pre filter and carbon filter assembly; D) Arsenic/phosphate filter manufactured by BASF and distributed by Aquasana® in Houston, Texas; E) Filtered water reservoir; F) Reconstitution barrel where minerals and salts are added back to the RO water; G) ProMist® mister motor that delivers spray to each of the enclosures in the room. (Photo: J. Pramuk)

14 Phos-Zorb®, Aquatic Eco-systems, Inc. phosphate sponges, and Poly Filters® work well for phosphate removal. Another option would be to use a Tide Pool® sump filter and to place a phosphate pad in it. Cycling the water with the same pad (i.e., having a closed system) will remove more phosphate from your water source over time. The

**Dissolved Oxygen**

Amphibian larvae absorb oxygen through their gills and skin and/or by gulping air. The amount of oxygen required by an aquatic amphibian depends to a great part on its natural history. For example, lentic (pond-dwelling) species require less oxygen than lotic (stream-dwelling) species. Dissolved oxygen (DO) is the amount of oxygen present in freshwater. Employing a filter and/or air tube with air stone to increase the amount of DO in the enclosure may be necessary for many stream dwelling species. However, the more we learn about amphibians and their complex natural histories, it is increasingly apparent that there are always exceptions to a rule. For example, centrolenid (glass frog) tadpoles, which drop from leaves upon hatching into moving streams, quickly find their way to stagnant pools off to the side where they encounter (and thrive) under low oxygen conditions (Hoffmann, 2010) and thus may not thrive under highly oxygenated conditions.

Freshwater is said to be fully saturated with oxygen when it holds the theoretical maximum for a given temperature and atmospheric pressure. The warmer the water and the lower the atmospheric pressure, the less oxygen water can hold. Dissolved oxygen concentrations must be sufficient to support the enclosure's aerobic community, including the amphibians, their food source, and the nitrogen-consuming bacteria (the tank's "biofilter"). DO concentrations are dependent upon the enclosure's water volume and surface area; stocking density and organic load; and the efficiency of the biofilter. Insufficient levels of DO (<80%) accelerate the decomposition of organic matter, releasing poisonous gases such as hydrogen sulfide (detectable by its signature sulfurous smell of rotten eggs and thereby serving as a warning of low DO levels), carbon monoxide, and methane (Odum and Zippel, 2004). DO levels can be increased by aerating and circulating the water by use of a standard air pump and air stone and should be monitored by using test kits or electronic meters. A simple and attractive way to increase DO is to use a water pump to create a water feature (i.e., waterfall).

Too much DO and other gases in the water can lead to supersaturation. Often municipal tap water, due to high pressure and temperature changes, has high levels of dissolved gases, which can come out of solution upon contact with submerged animals. If bubbles are apparent on the skin of the animals or on the surfaces of submerged objects in the tank, the water may be supersaturated. This phenomenon can lead to "gas bubble disease" which can lead to erythema, hemorrhage, disorientation, and death (Whitaker, 2001). Supersaturation of water can be prevented by placing the influent hose above the water's surface when filling a reservoir tank, aging the water, and bringing it to room temperature before use. Aeration also is important to break the water surface tension allowing the gases to dissipate. Utilizing an air stone and aquarium pump to aerate the water for a day or two prior to use will create equilibrium and reduce the chance of supersaturation.

**General Hardness**

General hardness (also called Total Water Hardness) is the amount of salts dissolved in freshwater and is measured through chemical titration to degrees of hardness (dGH) (Table 1) or via electrical conductivity in micro Siemens (μS) (Andrews et al., 1988). Primarily, minerals that contribute to hardness are calcium and magnesium, but also include copper, zinc, iron, boron, and silicon. Soft water contains up to 75 mg/L calcium carbonate ($CaCO_3$) while hard water, in comparison, contains 150–300 mg/L $CaCO_3$. In nature, rainwater usually is quite soft and species that live in microclimates fed by rainwater (e.g., ephemeral pools and leaf axils of plants) will do best in softer water. Generally, the water hardness for amphibians should not exceed 150 mg/L $CaCO_3$ (~8.5 dGH). Calcium and magnesium salts can be added to harden water or deionized, distilled, or reverse osmosis (RO) water can be added to soften water (Odum and Zippel, 2004).

effluent of the Tide Pool® filter should run through a strong ultraviolet sterilizer to kill bacteria. A powerful aquarium pump (e.g., 600 gallons/minute) is required to return filtered to return water to the tanks above.

| Water Softness (dGH) | Mineral Saturation (ppm) | Softness |
|---|---|---|
| 0–4 | 0 –70 | very soft |
| 4–8 | 70–140 | soft |
| 8–2 | 140–210 | medium hard |
| 12–18 | 210–320 | fairly hard |
| 18–30 | 320–530 | hard |
| >30 | | liquid rock |

**Table 1.** Degrees of general hardness and their corresponding water softness.

## Alkalinity

The alkalinity of water is a measure of the ability of a solution to neutralize acids or its buffering capacity.

## Nitrogen

Unlike most terrestrial organisms such as reptiles, which secrete concentrated uric acid or other relatively nontoxic forms of ammonia, nearly all aquatic amphibians (including tadpoles) excrete nitrogenous waste as ammonia. This mode of waste excretion is energy efficient however the waste product is highly toxic and is dependent on a healthy external environment to ensure safe ammonia levels. Ammonia has two forms in water: the highly toxic un-ionized ammonia molecule ($NH_3$), and the less-dangerous ionized ammonium ($NH_4^+$) (Odum and Zippel, 2004). Dependent on temperature and pH, aqueous ammonia and ammonium will exist in equilibrium:

$$NH_3 + H_2O \Leftrightarrow NH_4^+ + OH^-$$

The concentration of toxic ammonia ($NH_3$) will rise with increases in temperature and pH and will fall and be converted to the less-toxic ammonium ion ($NH_4^+$) as temperatures and pH decline. Most water quality tests measure the total ammonia nitrogen (TAN) as the total amount of ammonia plus ammonium, however pH and temperature will also need to be evaluated to determine the actual value of toxic ammonia in the water. Table 2 can be used to determine the percentage of un-ionized ammonia (i.e., the more toxic ammonia) in water at a given pH and temperature:

| Temperature (C) | pH | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Acidic* | | *Neutral* | *Basic* | | | |
| | **6.0** | **6.5** | **7.0** | **7.5** | **8.0** | **8.5** | **9.0** |
| **5** | 0.0125 | 0.0395 | 0.125 | 0.395 | 1.23 | 3.80 | 11.1 |
| **10** | 0.0186 | 0.0589 | 0.186 | 0.586 | 1.83 | 5.56 | 15.7 |
| **15** | 0.0274 | 0.0865 | 0.273 | 0.859 | 2.67 | 7.97 | 21.5 |
| **20** | 0.0397 | 0.125 | 0.369 | 1.24 | 3.82 | 11.2 | 28.4 |
| **25** | 0.0569 | 0.18 | 0.566 | 1.77 | 5.38 | 15.3 | 36.3 |
| **30** | 0.0805 | 0.254 | 0.799 | 2.48 | 7.46 | 20.3 | 44.6 |

**Table 2.** Ammonia relative to pH and temperature (adapted from Emerson et al., 1975).

Amphibian waste and uneaten food produce toxic waste products (ammonia and nitrite). Clinical signs of ammonia intoxication include color change, increased mucous production, erythema (i.e., redness of the skin), and lethargy (Whitaker, 2001). Long-term exposure to elevated ammonia levels can lead to a compromised immune function and secondary infection. Water tests should be performed weekly, and a thorough water analysis completed whenever an illness is suspected (see box to right). A mechanical filter, weekly partial water changes, and good aeration and circulation will help keep water quality in balance. In a pinch, ammonia levels also can be regulated by using an additive such as AmRid® or AmLock® but frequent water changes should always be the keeper's first choice for making needed water quality corrections.

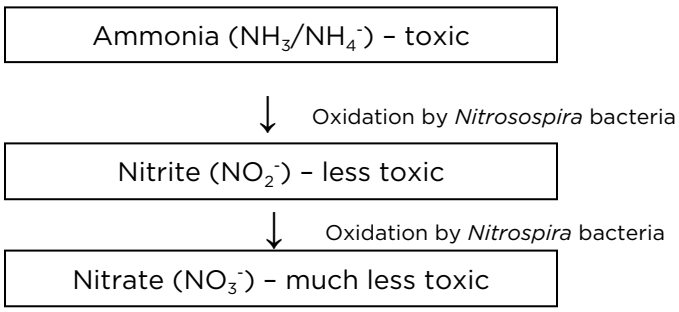
**Tracking Water Quality**

The following water quality measurements should be maintained by keepers in a logbook to track chemistry changes over time:

- Temperature
- pH
- Ammonia
- Nitrite
- Nitrate
- Hardness
- Alkalinity

Filters designed for fishes and available from any pet supplier work well for amphibians. A good filter will provide both mechanical removal of large organic matter and denitrification through biological processes. Chemical compounds such as carbon filters, phosphate sponges, ammonia-absorbing clays, or resins[15] may be used to target removal of specific chemicals. Several types of filters are available on the market and include types that employ polyester fiber, canisters or cartridges, or sand as methods of mechanical filtration. Air, rotary magnetic motors, or water pumps drive filtration mechanisms. After a few weeks or months of use, efficiency of all filters will decrease as debris inhibits water flow through the media. Regular cleaning schedules should be followed to ensure efficiency of the filter.

Just as they do in nature, beneficial bacteria in the terrarium work to convert ammonia toxins from waste products to less-toxic forms (nitrate and nitrite) through a process called biological filtration or biofiltration. Biological filtration takes advantage of naturally occurring communities of *Nitrosospira* and *Nitrospira* bacteria working in concert to oxidize reactions and convert toxic forms of nitrogen to safer forms. The process can be explained by following chemical reactions that are a portion of the nitrogen cycle:




An enclosure that is well established with denitrifying bacterial organisms will be a healthy microcosm that maintains waste levels naturally. Take care during the cleaning process to preserve the bacterial colonies by not using chlorinated water to clean the filter and by not being too physically vigorous in cleaning. Denitrifying bacteria live on all aquatic surfaces, and it is important to maintain this bacterial layer as much as possible to allow for recolonization of surfaces after cleaning.

15 Such as Zeolite® or Ammo-Chips®

The biofilter should be thought of as a living organism and requires two crucial things for proper health and maintenance: suitable substrate and ample oxygen. Appropriate substrates consist of ceramics or aquarium gravel and provide increased surface area upon which the nitrifying bacteria can thrive. The beneficial species of bacteria are aerobic, requiring sufficient oxygen to survive. Oxygenation is accomplished by circulating oxygenated water through the biofilter. Water should flow through the filter media slowly enough for the bacteria to absorb the nitrogenous wastes, but fast enough to maintain an aerobic environment. An anaerobic filter environment, created by slow or stagnant water, also produces ammonia and other toxic byproducts, such as hydrogen sulfide. This situation can be fatal to amphibians.

It is possible to set up a tank with external biofilters attached to it so that biologically active filters are ready to go at a moment's notice. Nitrogen consuming bacteria can be encouraged to grow by seeding the tank with household (non-scented) ammonia.

Ultraviolet light sterilizers work well for reducing levels of harmful bacterial in water. The ultraviolet bulbs lose effectiveness over time and should be changed every six months.

Water changes are crucial and should be performed as often as required by each particular system. There is no universal formula for frequency of water changes, as it depends on the number and size of animals in the enclosure, the volume of water, frequency of feeding, efficiency of the filters, and plants associated with each tank. Frequent water quality monitoring will help determine how often to change the water in any given system.

**pH**

The pH of water is basically the proportion of hydrogen ($H^+$) and hydroxide ($OH^-$) ions in solution. The pH scale is logarithmic with each pH unit representing a 10-fold change in the number of hydrogen ions. For example, a pH of 6 is 10 times lower than a pH of 7 and is 100 times lower than a pH of 8. When the number of hydrogen ions is greater than the number of hydroxide ions present, the water is acidic and its pH value falls below 7, conversely when hydroxide exceeds hydrogen, the water becomes basic (alkaline) with a pH value above 7. With a neutral pH of 7, hydrogen ions are equal to hydroxide ions. Natural pristine water sources generally have a pH between 6.5 and 8.5 (Ultsch et al., 1999) that can be dramatically influenced by air, water, or soil contamination. Many amphibians prefer a pH that is slightly basic. However, as pH requirements vary by species, a pH of 7 is recommended as a good starting point if the optimal pH is unknown. Some amphibians prefer slightly acidic water, such as aquatic caecilians and peat bog-breeding species such as Pine Barrens treefrogs (*Hyla andersonii)*. Salamanders that dwell in limestone aquifers, such as the Barton Springs salamander (*Eurycea sosorum),* require a slightly alkaline (basic) environment.

As indicated in Table 2 (above), the level of toxic ammonia ($NH_3$) is relative to pH and temperature. The ammonia produced by the accumulation of decomposing detritus and the products of animal and bacterial respiration progressively causes the pH to fall (become acidic) within an enclosure. The pH can be kept near neutral through regular cleaning of the mechanical filter, routine water changes, and good aeration. If a high pH requires immediate correction, acidity can be increased relatively safely in two ways: 1) add a commercially prepared blackwater treatment or 2) add tannins in the form of peat or sphagnum moss, placed within a tied-off piece of cloth or knee-high hosiery and set inside the tank (much like a bag of steeping tea). Adding reverse osmosis (RO), deionized, or distilled water will also lower the pH, however because these water sources do not contain buffering solutes the resulting pH may not be stable (Odum and Zippel, 2004). The system can be made more basic (raise the pH) by adding small amounts of sodium bicarbonate (baking soda) slowly to the system (approximately 1/8 teaspoon per 20 gallons of water), and wait for 24 hours prior

to testing and readjusting, if needed. When changing pH, go slowly to minimize impact on the physiology of the animals; aim for no more than a 0.5 pH change per 24 hours.

## Source Water Treatment

Reverse osmosis (RO) or carbon filters are the types of water purification most widely used by herpetoculturists to improve source water for use with amphibians. RO systems employ a semi-permeable membrane that selectively allows some atoms or molecules to pass through but not others. The membrane leaves behind impurities such as salts, but is not effective in removing some smaller compounds such as phosphate from the water. RO (and distilled) water is free of solutes and is purer than what can be tolerated by most amphibians. Its high purity (low level of solutes) means that in an attempt to reach osmotic equilibrium, water will move from the relatively pure surrounding water into the body tissues of an amphibian, which contain higher concentrations of solutes. Over time, this may result in edemic (bloated) animals and kidney problems. To compensate for the water's purity, salts and minerals should be added back to the RO water to create a solution that is isotonic (at equilibrium) with the physiology of amphibians (see sidebar for a reconstitution recipe). Pure RO (un-reconstituted) water is ideal for misting systems for display tanks where unsightly mineral deposits are not desired. However, inside the enclosure other sources (pools) of balanced water need to be accessible to animals to prevent osmotic imbalance within their bodies.

**Recipe for Reconstitution of RO Water**
(K. Zippel)

100 gallons RO water
15.0 g CaCl2 (Calcium Chloride)
17.6 g MgSO4 -7H2O (Magnesium sulfate)
13.6 g KHCO3 (Potassium bicarbonate)
11.3 g NaHCO3 (Sodium bicarbonate)
0.5 g commercial trace element mix

Dissolve crystals in a jar of water and add to storage vat. Blend thoroughly before use.

Final composition:
General Hardness: 3 degrees
Carbonate Hardness: 2 degrees
Ca:Mg (3:1)
Na:Ca+Mg+K (1:4)
pH ~ 7.4 depending on aeration

If in an area not prone to acid rain or excessive pollution, rainwater can provide a good alternative to municipal water. Rainwater collection barrels can be attached to downspouts on buildings, however copper, galvanized, or asphalt roof surfaces can contaminate rainwater with metals and other chemicals. Collection barrels are also open to contamination from amphibians that may dwell in gutters. Rainwater usually will be slightly soft and is particularly useful for keeping leaf axil breeding amphibians such as the strawberry poison dart frog [*Oophaga* (=*Dendrobates*) *pumilio*].

**Seven Commandments of Healthy Water**
(Odum and Zippel, 2004)

1. Start with high-quality water and test water quality parameters on a routine schedule.
2. Keep water fresh through frequent, partial, or complete water changes; proper flow-through, and/or filtration.
3. Clean mechanical filter media at least weekly.
4. Replace chemical media regularly.
5. Treat biological media as living creatures. They need oxygen and food (nitrogen).
6. Do not overcrowd or overfeed animals.
7. Incorporate living plants as much as possible.

## Water In, Water Out: How to Handle Wastewater

Wastewater disposal can be an issue when keeping non-native species, conducting disease research, or performing activities where it is necessary to reduce the risk of transmitting foreign pathogens to the local environment. Working with native species in their natural

range requires less consideration in terms of treatment of effluent for pathogens, as long as other biosecurity measures are followed (Pessier and Mendelson, 2010). In cases where wastewater is treated thoroughly by municipal facilities with chemicals (e.g., chlorine) and other methods before it is returned to the environment, additional treatment prior to releasing amphibian waste into the sewer is unnecessary. Feces and other biomaterials resulting from amphibians in quarantine should be incinerated or treated with other biohazard materials. In situations where municipal water treatment is not established or there are other risks of a foreign or novel pathogen being released into the environment via wastewater effluent from the facility, it is critical that all wastewater is appropriately treated and disinfected prior to disposal (see Pessier and Mendelson, 2010 for a thorough discourse on this subject). If lingering doubts remain about how to treat amphibian wastes and wastewater effluent, it is recommended to discuss this topic with staff veterinarians.

## ENVIRONMENTAL CONDITIONS

The large diversity of amphibian species is a result of the variety of habitats they have successfully colonized and the broad range of environmental parameters in which they live. Accommodating amphibians will require attention to temperature (air and water), light, and humidity, which can vary significantly between specialized, natural habitats and microhabitats. Additionally, diligence for replicating naturally occurring annual temperature, humidity, photoperiods, and rain cycles may be key for the captive-survival or reproduction of some species.

### Air Temperature

Maintaining appropriate temperatures for amphibians is one of the most important considerations for their overall health. As ectotherms, amphibians are unable to produce substantial amounts of body heat and instead rely on environmental temperatures and behavioral modifications (e.g., basking or hibernation) to meet their thermal requirements. Temperature requirements for amphibians are as important as they are for reptiles or fishes. A popular misconception is that all amphibians need to be kept cool, but this is where natural history research is important. If keeping tropical montane species, their median temperature requirements will be lower than tropical lowland species. Invest in an infrared temperature gun and frequently monitor the temperature gradient throughout the enclosures, making sure temperatures are within the range acceptable for the species.

The microhabitat of each species' native habitat should be considered. Some amphibian species live in microhabitats that are different from that expected of the geographic area in which they live. For example, temperate species usually do well at temperatures in the range of 18–24 C (65–75 F); however, cooler temperatures or a brumation period may be required for the autumn and winter seasons. Tropical lowland frogs can be maintained at 75–85 F (24–30 C). Tropical montane frogs generally do well when kept from 65–75 F (18–24 C) (Whitaker, 2001). Eggs and larvae of most Neotropical hylids and dendrobatids can be maintained from 77–80 F (25–27 C) (Cover et al., 1994; Whitaker, 2001). Aquatic caecilians (of which all species are tropical) can be kept at relatively high temperatures from 80–85 F (27–30 C).

It is usually easier to add heat to an enclosure than to remove it. It is therefore recommended to maintain amphibian rooms slightly cooler than the average temperature required by collection animals. Heating individual enclosures with basking lights or submersible heaters to create thermal-gradients allows specimens to move about the enclosure in order to regulate their internal body temperatures. Ideally, it is preferable to alter the temperature of the entire room rather than using heat pads or heat lamps to regulate temperature for each enclosure, as these methods can dry out enclosures quickly, unpredictably affect humidity levels, or overheat animals which can quickly lead to death. However, this luxury usually is not viable with cosmopolitan collections requiring a wide range of temperature regimes that are housed together in a single room.

If amphibians are confined to a small room or greenhouse-like structure, a portable room AC/heating unit can be installed to regulate the ambient temperature.[16] These units are rather expensive (~$700) but have been used at Omaha's Henry Doorly Zoo to maintain temperature in small rooms with great success (See Chapter 5).

**Water Temperature**

Water temperature will generally mirror the ambient temperature of the enclosure. Make certain that incoming water has reached room temperature prior to use on animals. A good way of ensuring water temperature equilibrium is by having a reservoir to acclimate the water prior to use. Depending on conditions, heat or cool the water in a reservoir or within an aquatic enclosure. Remember to keep any reservoirs free of contaminants. As a general rule, choose a submersible heater that can provide 5–10 watts of output per gallon of water being heated. Water heaters are fairly inexpensive (~$15), while chiller units for aquariums are relatively expensive (~$700). Water chillers, however, are particularly important for culturing coldwater salamanders such as hellbenders (*Cryptobranchus* spp.).

**Lighting**

While much more research is needed to document the specific requirements and benefits, quality and quantity of light are both important to amphibians. Some species require ultraviolet light for calcium metabolism, normal behavior, and reproduction. Amphibians (and reptiles) manufacture vitamin $D_3$ from exposure to ultraviolet-B (UVB) radiation through a process in which Vitamin $D_2$ is converted to $D_3$. Vitamin $D_3$ is critical for proper absorption of the calcium necessary for building and strengthening bone. Many species of frogs such as harlequin frogs (*Atelopus* spp.) and some leaf frog species (*Phyllomedusa* spp.) bask regularly and thus would seem to require a stronger source of ultraviolet light than many other species. Even non-basking species receive some reflected ultraviolet light; it is important to their physiology and development as well.

The sun emits two types of ultraviolet radiation relevant to herpetoculture: ultraviolet-A (UVA) and UVB. UVA includes long-wave solar rays of 320–400 nanometers (billionths of a meter). UVB radiation is comprised of short-wave solar rays of 290–320 nanometers. Both types of ultraviolet radiation can cause point mutations in DNA and in excess doses can cause cancers or other problems. In humans, UVB rays are more potent than are those of UVA in producing sunburn. Natural light is by far the best option for captive animals, but is not always available. In lieu of natural daylight, timers can be used to maintain the ambient photoperiod in a natural cycle. Because glass does not transmit middle-wavelength ultraviolet light, it should not be used for cage tops; instead, choose wire mesh. Acrylic and fluoroplastics transmit some short wavelength ultraviolet light (Gehrmann, 1987), but wire mesh is best.

Artificial lighting has come a long way towards replicating the real thing, but it is still far from replacing natural light. Lights should be placed a sufficient distance from animals so as not to cause burns or other problems, but close enough for the ultraviolet to be effective. The UVB output of fluorescent bulbs decreases substantially after a few hundred to a few thousand hours of use. Unfortunately, the gradual change in ultraviolet-effectiveness is not apparent to the human eye; brands, and even batches, of bulbs may vary in their UVB output. In addition, the nature of enclosure lids and screens, as well as the distance to the bulbs, will impact how much UVB is actually available to the animals inside. Obtain bulb-life information from the manufacturers and periodically measure ultraviolet-output with a UV meter[17] to monitor bulb-

16 Such as the Sunpentown® 12,000 BTU unit
17 Such as SolarTech's solarmeter® UV(A+B) model 5.7 or UVB model 6.2

life, changing them accordingly. Use of a UVB meter determines UVB strength and allows for experimentation with new bulbs, screens, etc.

Many ultraviolet-emitting lights are now available on the market and have been designed specifically for amphibians and reptiles.[18] Note that there are very few data indicating the precise UVB requirements for amphibians, nor how species may differ in their ultraviolet uptake (Pough, 2007). However, there is some anecdotal evidence that ultraviolet lighting has improved amphibian husbandry, especially the health of diurnal species. For example, modified Eiko® M16 halogen bulbs (Eiko® Supreme Ext/Su/10K, 12V 50W; with the lens carefully removed using a Dremel® tool and pliers) are widely used by several zoos and provide full-spectrum ultraviolet light. Evidence suggests that these lights allow a 5- to 20-fold increase in the conversion of vitamin $D_3$ from UVB among some amphibians, compared to other light sources (Browne et al., 2007). The bulbs last more than two years, cost less than $4 apiece and therefore are more cost-effective than many brands of reptile bulbs currently available. Track-light kits compatible with these bulbs can be purchased from online stores listed at the end of this chapter (Figure 16).


**Figure 16.** Left, a terrarium set up for Kihansi spray toads (*Nectophrynoides asperginis*) custom manufactured by All Glass Aquariums. This tank is on an open system and is affixed with mist nozzles and full spectrum lighting. Right, Eiko® bulbs and tracks used for lighting amphibian enclosures. (Photos: J. Pramuk)

## Humidity

Relative humidity (RH) is the percentage of water vapor a given volume of air or the proportion of water in the atmosphere relative to the amount of moisture the atmosphere can hold. It is a relative measure because the absolute value changes with the air temperature. In other words, warmer air will contain more water vapor than will cooler air with the same RH, thus a direct correlation with temperature.

Humidity is extremely important because it will determine how quickly an animal will lose water from its body to the surrounding air. Lower humidity, such as in a desert environment will ensure that water is lost at a faster rate. For this reason, many amphibians will only become active at night, during rainstorms, or during rainier times of the year.

Humidity levels in rooms will fluctuate depending on the time of year. In warmer months, if air conditioners or forced heating are used, the RH will decrease and moisture will be more rapidly lost from enclosures and animals. Humidifiers can be used to increase RH within a room. Many types of room humidifiers are available commercially and are relatively inexpensive ($30–150). Some portable humidifiers have been reported to release

18 Some of these brands include ZooMed® ReptiSun®, Coralife® Incandescent Reptile Bulb, etc.

microorganisms and minerals from their reservoir into the air, which can lead to health problems. Avoid using steam vaporizers for amphibians, as they generate high heat. Evaporative humidifiers are inexpensive to operate, but they need to be kept clean of scale deposits (accumulated minerals) which will build up in the water reservoir and create problems. Although expensive at approximately $1/gallon, distilled water can be used to fill evaporative humidifiers, greatly reducing the release of microorganisms and mineral deposits into the air. Ultrasonic humidifiers are expensive initially and to operate but are not prone to the same level of bacterial build-up as evaporative models.

While maintaining ambient humidity in a room is helpful, what is more crucial will be maintaining humidity within an enclosure containing amphibians. There are many ways to add or regulate humidity within enclosures ranging from automatic misting systems to hand spraying to regulating the amount of ventilation. Automatic misting systems are excellent for maintaining constant levels of humidity within large numbers of enclosures and come in a variety of sizes, brands and configurations. Although labor intensive, hand misting with small hand sprayers or larger "pump" sprayers allows more opportunities for observation by the keeper and can aid in overall monitoring of animals.

**Figure 17.** Cool-mist humidifiers offer another way to achieve a sudden increase in humidity that may stimulate breeding. (Photo: R. Gagliardo)

Breeding behaviors in some species may be stimulated by increases in humidity, rather than heavy rain. Simulate this by venting a humidifier into the tank with PVC pipes or flexible tubing (Figure 17). Sealing the tank with plastic wrap will help hold in the humidity, although small air vents should be included to allow for dissipation and fresh air exchange.

**Tools**

Prior to placing animals in an enclosure, monitor the environmental parameters every few hours for a few days. It is very easy to overheat animals so ensure that the highest temperatures fall within the acceptable range for the species. There are a number of tools that facilitate the monitoring of conditions.

Digital and analog thermometers are essential for measuring the temperature both inside and outside enclosures. A simple maximum/minimum (max/min) or digital thermometer (Figure 18) serves well for obtaining basic information on daily temperature fluctuation. Infrared temperature guns are ideal for measuring the temperature inside an exhibit or enclosure


**Figure 18.** Digital thermometer. (Photo: J. Pramuk)


**Figure 19.** A HOBO® temperature and humidity data logger. (Photo: J. Pramuk)

without disturbing the animals or opening the enclosure. Many newer models also measure humidity in addition to temperatures. For detailed records, portable data loggers[19] work well for measuring environmental conditions within enclosures or amphibian rooms (Figure 19). These digital devices can measure RH, temperature, and even light levels. Depending on the model, up to 65,000 measurements can be stored. These devices come with an easy-to-use software program that allows intervals to be logged, start times selected, and recorded data to be downloaded to a spreadsheet program such as Microsoft® Excel to produce detailed charts and graphs.

**FOOD**

In nature, frogs, salamanders, and caecilians have the benefit of eating prey items fortified with naturally-occurring nutrients, the effects of which is poorly studied in amphibians. Malnutrition of amphibians can lead to developmental (e.g., the dreaded spindly-leg) and reproductive problems, metabolic bone disease, tetany and paralysis, failure to thrive, and death. Keep these issues in mind when selecting among the following three sources of food items: commercially available, wild caught, or cultured. Regardless of the source, it is best to offer a diet that closely resembles what the species would eat in the wild, along with the greatest variety possible. Keeping amphibians is almost secondary to producing or acquiring adequate and nutritious prey items, and as biologists one should strive for improvements in this area.

Important things to consider when selecting food for animals are: 1) the calcium:phosphorous ratio; 2) lipid (fat) content; and 3) size of the prey item. Frequency of feeding also is critical and will depend on the natural history of the species being maintained. For example, dart frogs are highly energetic and require frequent feedings (at least three times per week). A good rule to use for energetic species is that there should be insects remaining in the enclosure between feedings so that the animals can feed *ad lib*. More sedentary species such as some members of the frog genera *Ceratophrys, Dyscophus, Litoria,* and *Pyxicephalus* and salamanders such as *Ambystoma* are prone to obesity and feedings should be monitored accordingly. Avoid feeding too many fatty prey items (e.g., pinky mice and waxworm larvae) to these and other ambush predators as their metabolisms are slow. A good vitamin supplement with a calcium:phosphorous ratio close to 1:1 should be offered several times a week with feedings. It is critical to consider the natural history of the species in deciding how and when to feed. Feedings should be based on the animal's natural feeding times, rather than on the convenience of the keeper's schedule. Feeding nocturnal species early in the day can give the food items time to hide and elude predation before the lights go off. Using feeding bowls or hand-feeding techniques for critical species can aid monitoring health and proper nutrition.


**Figure 20.** Examples of commercially-prepared vitamin supplements suitable for amphibians. (Photo: J. Pramuk).

**Nutrition**

There are several reptile and amphibian vitamins on the market that are quite good including Reptocal® mixed 1:1 with calcium carbonate ($CaCO_3$), Reptivite®, and Nekton® products, etc. (Figure 20). More recently developed products (e.g., Repashy Calcium Plus®) are apparently producing good results for many herpetoculturists but these reports are mostly anecdotal. The FDA does not test and regulate animal vitamins,

19 Such as HOBO® (manufactured by Onset) or Logtag® TRIX-8

therefore the percentages of vitamins and minerals in these products are not monitored and therefore, there is no guarantee of what is in the final product. One way of circumventing this problem is by using human vitamins.[20] Vitamin supplements degrade quickly at room temperature and with exposure to light. Therefore, it is recommended that vitamins are purchased in small batches. Unused quantities should be refrigerated with aliquots used over a period of no more than a month.

**Vitamin A**

Recently, the role of Vitamin A has become a hot topic in amphibian medicine. While it is not completely clear how amphibians utilize Vitamin A, deficiency in this nutrient has been associated with lingual squamous metaplasia ("short-tongue syndrome") in Wyoming toads [*Anaxyrus (=Bufo) baxteri]* (Pessier, et al., 2002.). Further anecdotal information indicates that Vitamin A may have roles in maintaining healthy immune systems, reproductive viability and other significant aspects of amphibian health (see McWilliams, 2008; McComb, 2010 for more information). Use supplements with fully-formed *Vitamin A* rather than *beta-carotene* (Vitamin A), as the latter is apparently not available as a Vitamin A source to insectivores and at least one study suggests that carotenoid precursors (beta-carotene) may not be converted within the amphibian body (McComb, 2010).

## Culturing Food Animals

The advantage of culturing live foods is that it gives the keeper control over the cleanliness of their cultures. Often this will be the most cost-effective method of obtaining insects. It is time-consuming and labor-intensive, and in the case of some colonies, quite odiferous. If possible, catching appropriately sized insects from the wild is a great way to supplement animals' diets; however, this may introduce parasites and chemical contaminants such as pesticides.

**Insect Collecting**

Many insects can be collected locally by sweeping butterfly nets in tall vegetation. Other effective methods of collection include employing insect traps at night that are attached to black or mercury vapor lights.[21] However, ensure that the collection area is chemical-free (i.e., pesticides or herbicides) and keep in mind that using wild-collected insects hypothetically might increase the risk of introducing pathogens or parasites.

One easy way to collect nutritious insects is by trapping termites, a commonly available and nutritious food source in many areas of the world. Roll moist, pesticide-free corrugated cardboard and place into a two foot (0.6m) long piece of PVC pipe with a cap on one end. Drill some holes into the lower half of the pipe. Bury the open half of the pipe into the ground, preferably near a termite-colonized tree or log. After a week or two, periodically check the trap for termites. If occupied, shake the open pipe end into a bucket to collect termites. Animals that particularly relish termites include natural ant consumers such as microhylid, dendrobatid, and mantella frogs.

**Crickets**

Crickets, as well as mealworms (beetle larvae), spikes (house fly maggots), mousies (crane fly larvae), and reportedly high-calcium soldier fly larvae (e.g., Phoenix Worms®) can be purchased from U.S. suppliers. Some of the calcium reports should be considered carefully, as the raw calcium content of prey does not relate directly to availability for the amphibians (Dierenfeld and King, 2008). In other words, the prey's calcium may remain unavailable to the amphibian.

20 Such as One a Day Men's Plus®

21 These types of traps and butterfly nets are available from BioQuip®.

The most common insect used to feed amphibians in captivity is the domestic cricket, *Acheta domestica.* Crickets can be cultured on-site or purchased from a bait or animal food supplier. Usually, suppliers provide various sizes suitable for feeding animals. Hatchling crickets (pinheads) are appropriate for feeding dart frogs, mantellas, and smaller species of salamanders. Adult crickets are suitable for larger species of amphibians such as larger ranids [e.g., gopher frogs such as *Lithobates (=Rana) capito*], and bufonids [e.g., the cane toad *Rhinella* (=*Bufo*) *marina*], and *Dicamptodon* salamanders. Crickets that are too large for an animal will either not be consumed, or can rupture the digestive tract of the amphibian that is able to swallow something larger than it can accommodate. Crickets can be housed in a plastic utility sink or garbage can, available from hardware stores (Figure 21). Use a layer of petroleum jelly in a ring or a band of smooth packing tape around the top of the enclosure to minimize cricket escapes from the enclosure. Provide stacked cardboard egg crates to increase surface area for crickets. Dietary moisture may be provided via a chick feeder[22] or sliced fruits or vegetables (i.e., oranges, fresh squash, dandelion, kale, endive, turnips, carrots, yam, and/or beets) on a rotating basis for variety, supplemented with dry fish flakes. Crickets do not have an optimal calcium:phosphorus ratio and should always be gut-loaded with a high-calcium cricket diet[23] for a minimum of 48 hours prior to feeding to animals. Gut loading is the practice of feeding, or "loading" food animals with a nutritious food prior to feeing them out to a predator. Gut-loaded crickets are closer to the ideal calcium:phosphorus ratio of 1.5:1 (Wright and Whitaker, 2001). They also should be dusted with a premium vitamin powder immediately prior to feeding out. There is preliminary evidence that American toads *[Anaxyrus (=Bufo) americanus]* fed crickets that were gut loaded with a vegetable mixture of three equal parts yam, sweet potato and kale as opposed to commercially available grain-based gut loading powder produced toads with higher Vitamin A levels (Odum et al., in prep.).

**Figure 21.** Plastic laundry sinks make useful enclosures for cricket colonies. (Photo: R. Gagliardo)

Crickets can be cultured easily by offering an adult colony a shallow plate or tub filled with a mixture of moist sand and sterile soil or peat moss in which the females may oviposit (lay their eggs). After three or four days, the egg filled container can be removed and place d into an aquarium. The eggs are small, white, and ovoid (appearing like tiny grains of rice). Ten to fourteen days later, the pinheads should hatch. Some institutions place breeding tubs in with newly purchased adult crickets during the gut-loading time period to increase culturing potential.

To separate the pinhead crickets from the soil substrate, place the container filled with eggs on a piece of egg crate that is suspended across an aquarium. When the pinheads hatch, they will fall through the egg crate into the clean enclosure below. Use a moist sponge on a plate, inverted test tube with a sponge on one end, or perhaps daily deliveries of fresh greens, oranges, or squash to provide water to the pinheads. Make sure crickets are maintained at moderately high temperatures 80–85 F (27–29 C) at relatively low humidity. A maintenance diet of high-calcium feed or chicken scratch can be used to feed growing crickets. It is essential to keep the cricket colonies clean to reduce bacterial problems and growth of phorid flies, a pesky, opportunistic species that can infect cricket colonies and reduce

22 Available from feed stores or the internet.
23 Such as Mazuri® high-calcium feed or Zeigler's® Hi-Cal cricket diet

productivity. Frass (insect feces) and exoskeletons should be removed every other day or more depending on density.

As with all insects, place a rock or other small emergent object in all amphibian water dishes so that crickets will not drown. Moreover, be aware that crickets have been known to chew on the skin of amphibians. Placing a bottle cap with cricket feed in a terrarium will help prevent hungry crickets from injuring amphibians.

**Fruit Flies**

Fruit flies are a convenient food source for smaller and recently metamorphosed amphibians however, their calcium:phosphorous ratio [approximately 0.13:1 (or 0.13)] is not better than most cultured insects (Oonincx and Dierenfeld, 2011). Culturing fruit flies takes practice and a bit of finesse, but the concept is straightforward and once mastered, cultures can yield thousands of flies within a couple of weeks. Yeast grown on a sugar/starch source produces a sugar-alcohol that in turn supports the complete life cycle of the flies. A dry potato-based medium is hydrated in the bottom of a container by adding an approximately equal part of water (by volume) to the medium (Figure 22). There are two different species of fruit flies commercially available for culturing from biological supply companies: *Drosophila hydei* (the larger of the two species) and *Drosophila melanogaster*. Both species are sold as wingless or apterous (mutant) forms, making them easier to culture and for amphibians to catch. If both species are cultured on-site, raise them separately and do not mix them together. Fruit fly culture medium can be purchased commercially or made out of readily available ingredients (see blue box for two recipes).

**Figure 22.** Mature fruit fly cultures. *Left side*: Made from a jar. *Right side:* Made from plastic deli container. Breathable lids created using foam plug or filter floss inserted into a hole in the lid. Increase surface area inside the container by using cardboard, excelsior, or a paper towel. Both contain potato-flake-based instant fruit fly media. (Photo: J. Pramuk)

**Recipe 1: Fruit Fly Culture Medium**
(C. Eser)

Mix the following dry ingredients together:
3 cups instant potatoes flakes (without butter or other flavor additives)
2 teaspoons brewer's yeast
Mix the following wet ingredients together:
4 cups of boiled water
2 teaspoons molasses

Add dry and liquid contents together and stir. Divide the mixture equally into containers (e.g., one liter plastic deli containers or Ball® jars). Sprinkle top of each mixture with methyl paraben (a commercially available preservative) and brewer's yeast. Use about one teaspoon per container. For the brewer's yeast, add approximately 1/2 teaspoon per container.
Yield: enough for 6 one-liter containers.

**Recipe 2: Fruit Fly Culture Medium**
(R. Gagliardo)

Dry mix: 28 oz potato flakes, 3 cups powdered sugar, 8 oz brewer's yeast
Liquid mix: 1/2 and 1/2 water and white vinegar

By volume, mix 1 part dry mix and 1 part liquid. Sprinkle about 10 grains of baker's yeast on surface. Rinse sides of container with water and while doing so, wet the baker's yeast. Wait 2 minutes for initial ethanol release from hydrated yeast to dissipate, then inoculate surface of the mixture with a solid layer of fruit flies.

There are consistent, commercially available fruit fly media that come in white or blue.[24] They are identical except that blue dye is added to aid in seeing larvae as they burrow in the substrate. Antifungal agents are incorporated into the mix already, but if the cultures are kept in hot, humid conditions (or if cultures suddenly become moldy during the warmer summer months), it may be necessary to add a little additional methyl paraben or other food preservative to each culture. In addition to maintaining stable temperatures for cultures, it is imperative to maintain good hygiene in the area where cultures are stored. Avoid shelving made of wood or other porous materials that may attract and harbor mites which can easily invade and contaminate fly cultures. Keeping cultures on non-porous shelves (metal, glass, or plastic) or in shallow plastic trays that can be easily sanitized will be of great help. Label each batch of cultures with the *date created,* and use should be rotated regularly; cultures last for approximately one month after which time they should be retired. Clean jars with hot water and disinfectant, rinsed thoroughly, and allowed to dry before making new cultures, If cultures crash at facilities in the U.S. or Canada, it is possible to order new cultures from insect suppliers. Ed's Fly Meat offers a reduced rate to facilities with a standing order ("Monthly Meat"), although this is an expensive option. Culturing techniques are described in the blue box that follows.

**Fruit Fly Culturing Technique**
(I. Hiler)

1. Harvest flies from active cultures into a mason jar. Make sure jars used for new cultures are clean and dry. Cover with a secure lid. Select healthy cultures that are fungus and mite free. Harvest enough to add approximately 50 flies per new jar. Set aside.
2. Clean off workspace with disinfectant and dry thoroughly. Set up jars. Put 1/3 cup fruit fly medium at the bottom of each jar.
3. Add 1/3 cup (or slightly more) water to each jar. There should be no dry flakes left (dry flakes indicate that not enough water has been added).
4. Sprinkle a small amount (approximately 1/8th of a teaspoon each) of fresh yeast and vitamin powder on top of the moist medium. To keep them fresh, both of these additives should be stored in a fridge. Note: The yeast provided with the medium is usually stale and should not be used. Too much yeast will create an excess of CO2 and kill the colony.
5. Add a piece of slightly crumpled, moist paper towel (approximately 1/3 of a sheet) or a moist coffee filter on top of the medium (unbleached paper is preferable). This will allow more surface area for the flies and their offspring.
6. Sprinkle approximately 50 flies into the jar and immediately place a clean piece of muslin or coffee filter on top and close with threaded brass ring lid to prevent flies from escaping.
7. Place jars on a shelf, set in a warm area clear of draft, and labeled with a date.

**House Flies**

While conventional feeder insects such as domestic crickets and mealworms are readily available to amphibian culturists, there are other insects that can be easily cultivated and provide a quality and varied amphibian diet. House flies (*Musca domestica*) and their maggots are particularly relished by active hunters, such as treefrogs, and the flies can be easily reared out from maggots purchased from any bait company. House fly culturing is a time-intensive and often smelly process, but they can be reared using sugar and powdered milk to feed adults, and a dog food mixture for egg deposition and larval growth. An example protocol for rearing and breeding house flies is available at: www.spiderpharm.com/docs/housefly_kit.pdf

Purchased maggots are usually shipped in their last (3rd) instar and will pupate within a matter of days at room temperature. Once the flies emerge from their pupae, they can be refrigerated for up to a few weeks. Refrigerated flies will be sluggish and easier to feed out to

24 Such as Formula 4-24®, available from Carolina Biological Supply™

frogs. Dividing the chilled flies into smaller quantities in plastic test tubes (vented) is a good way to "grab and go" before feeding time. Dust cold flies with vitamin powder and then feed them out quickly before they resume flying.

**Mealworms**

Mealworms are commonly available from most insect suppliers. There are two commonly available species, *Tenebrio molitor* (a smaller larva) and *Zoophobas morio* ("giant" mealworms or "super" worms). Both are the larval stage of tenebrionid beetles, which are hearty, easy-to-keep insects. Larvae can be maintained in cricket meal or bran in a large plastic open container (e.g., a plastic dishwashing pan). To prevent escapes, use packing tape or petroleum jelly in a band around the top of the container. Slices of apple and/or potato provide good sources of moisture. Adult beetles will lay eggs in the substrate and the next generation will start anew. The larvae can be easily harvested from the media by using a kitty litter scoop or similar device. The media will need to be replaced once it has been consumed by the larvae and been replaced with their powdery frass (insect feces). Many people are, or become, allergic to mealworm frass, so wearing a dust mask is recommended when cleaning out the enclosure. Larvae can be harvested manually or with forceps. Note that these beetle larvae have a thick exoskeleton, which is difficult for many amphibians to digest. Adult giant mealworms should only be fed to the largest species of amphibians [e.g., African bullfrogs (*Pyxicephalus adspersus*)] and it may be beneficial to clip off their sharp, powerful mandibles prior to offering them to amphibians. Additional information on rearing mealworms is available elsewhere (Nehring, 1996).

**Bean Beetles**

Bean beetles (*Callosobruchus maculates*) are a small coleopteran that is easily cultured on dry legumes including black eyed peas, mung beans, chick peas (i.e., garbanzo beans) or adzuki beans (Figure 23). This insect is considered an agricultural pest in Africa and Asia, so it should be considered a potentially invasive species. Bean beetles are small (approximately 2 mm in total length) and are readily eaten by many smaller species of amphibians. In the United States, it is a model laboratory organism used for genetics and other scientific studies and can be purchased from biological supply companies such as Carolina Biological Supply. It is suggested that organically-grown beans be used if possible, to reduce the potential negative effects of pesticides on the beetles. Place about an inch or two of beans at the bottom of a 32-oz. deli cup with pinholes made in the lid for gas exchange. Add some beans containing beetle eggs on them from an older culture to the new culture. Maintain the culture at 71-86 F (22–30 C). Cultures raised at 77 F (25 C) will have a generation time of 5–6 weeks (Beck and Blumer, 2011). Higher temperatures will decrease generation time. The adults do not require food or water and will lay their eggs directly on dried beans. Bean beetles can be collected from the cultures by placing corrugated cardboard or toilet paper tubes inside the culture. Adults will climb up the cardboard, which can be lifted from the culture and shaken over a collection cup or vial where they can be dusted with vitamin powder prior to feeding to amphibians.

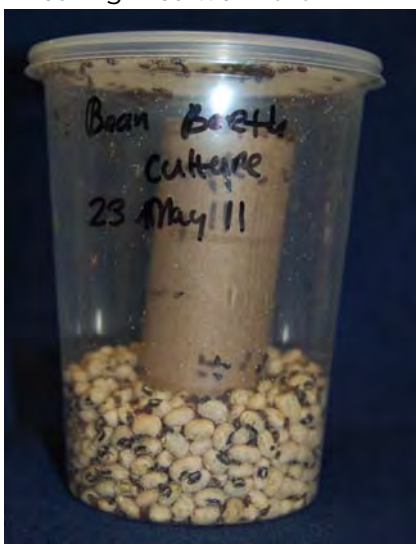



**Figure 23.** A mature bean beetle culture. The beans at the bottom of the deli cup are black eyed peas although adzuki, mung, and garbanzo beans work as well. The cardboard tube helps with beetle collection. (Photo: J. Pramuk)

**Pea Aphids**

Pea aphids (*Acyrthosiphon pisum*) are a common insect belonging to the order known as the "true bugs" (Hemiptera) and are being cultured with increasing regularity by amphibian culturists. As these are tiny insects, they make an ideal food for small species or neonates.

Aphids feed on plant phloem or sap and therefore can only be raised on living host plants rather than on an artificial diet. Each species of aphid is specific to a species or suite of plant species, and they feed by extracting sap from the terminal leaves or the stem of the host plant. Therefore, research which host plant is required for the type of aphid selected to culture. Pea aphids are a model laboratory organism and thus have been reared for many decades in laboratories. Pea aphids feed on a variety of bean or legume (Fabaceae) species and can be cultured on bean sprouts that are grown inside plastic containers such as a 32- or 16-oz. deli cup. To sprout the beans, place them on top of a moist layer of paper towels at the bottom of the container and set the container in a dark place. Once the beans have sprouted, introduce the aphids to the deli cup and inoculate new cultures with aphids every other week to maintain the colonies. In the U.S., "starter" aphid cultures can be purchased from Berkshire Biological. The North American pea aphid can be grown on fava bean sprouts, which can be obtained from online vendors[25]. Aphids also can be reared on young or adult legume plants grown in a green house. The aphids can be collected from the culture by tapping or shaking them off of the tips of their host plants. As aphids drown easily, avoid feeding them to your amphibians in enclosures with active misters, etc.

**Isopods**

Isopods (woodlice, pill bugs, etc.) are not insects but rather a type of crustacean. Terrestrial forms are commonly found beneath logs or rocks and the more than 10,000 species assigned to this order are nearly cosmopolitan in geographic distribution. They have seven pairs of legs and a segmented, dorso-ventrally flattened body. Common names include woodlice, pill bugs, sow bugs, or roly-polys. Woodlice (*Porcellio scaber*) contain one of the highest calcium:phosphorus ratios of any feeder insect [14.38:1.22 (or 11.79) vs. 0.13:1 (or 0.13) for domestic crickets] (Oonincx and Dierenfeld, 2011). Isopods can be cultured easily by keeping them in plastic 16-oz. deli cups or in similar plastic containers with tight-fitting, ventilated lids. The isopods should be maintained on a few inches of moist coconut fiber with a layer of dried leaves and corrugated cardboard on top of the culture. The substrate should not be too wet nor allowed to dry out. The isopods may be harvested by removing a piece of cardboard from the culture and shaking it over an open collection container. Supplemental food such as fish flakes or algae wafers can be added to the culture although the isopods can survive eating dried leaves and cardboard. There are many types of isopod available (e.g., dwarf whites, Spanish orange, and common gray) to purchase, including both temperate and tropical species. Choose a species that will do well in the ambient temperature of the invertebrate culturing room. Starter cultures of isopods are available from biological supply companies.

**Wax Moth Culture Medium**
(I. Hiler)
16 oz. Gerber Dry Cereal®
16 oz. dry oatmeal
32 oz. honey
4 oz. glycerin

Put dry cereal and oats in a blender or food processor and grind into a fine powder. Put the powder in a large mixing bowl with glycerin. Add honey and hand mix to incorporate all ingredients until a sticky-dough forms. To start a colony, add a dozen or so late-age larvae or moths. Their life cycle is about ten weeks. Usable worms for small frogs will be available from the culture for about three and half weeks. Yield: approximately 30 portions.

**Wax Moth (wax worm) Larvae**

Wax moth (*Galleria mellonella*) larvae are parasites of beehives and eat the wax and honey of the hive (see sidebar for recipe). Wax moth larvae are a very rich source of lipids (fats). They are good to feed to animals that are underweight or those being bulked-up for breeding. Do not over-feed wax moth larvae, as too much fat can lead to health problems such as lipidosis, which is often manifested in amphibian eyes as fatty deposits on the liver or fatty opaque deposits on the lenses of the eyes.

25 Such as Sun Organic Farm

**Roaches**
Roaches can be easily cultured in a well-secured aquarium by placing a layer of newspaper on the bottom and providing cork bark pieces for increased surface area. The roaches are fed a diet of vegetables and fruits, and although they are relatively slow to reproduce, they provide a nice alternative to crickets. Three species are currently propagated widely as amphibian food items:

**Lobster Roach (*Nauphoeta cinerea*)**
Probably the most commonly-bred of the feeder roach species. Adults are comparable in size to large crickets 1 inch (23–26 mm), although they have a greater "meat" to exoskeleton ratio than adult crickets. Adults of both sexes are winged, but flightless. Lobster roaches can easily climb glass, so measures to keep them contained should be employed. A 1–2 inch (2-5 cm) wide band of petroleum jelly or a product called Bug Stop®[26] will work perfectly well. This species is a very prolific breeder with a short time between generations. A newly hatched nymph can reach breeding age within three months. Adult females produce clutches of 20–30 nymphs at 30–60 day intervals. The female produces an ootheca (egg case); however, she pulls it back into her body for incubation. Individual roaches can live for 12–24 months.

**Discoid Roach (*Blaberus discoidalis*)**
The discoid roach is also easily propagated. The adults 1.4-1.8 inch (35-45 mm) are ideal for larger species of amphibians, but the nymphs are useful for smaller and medium-sized species. Both sexes are winged, but flightless, and they cannot scale smooth surfaces like glass. This species is not as prolific as the smaller lobster roach and colonies can take some time to become established; however, once they are established they can be quite prolific. Breeding age is reached within 4–5 months and the life span is 12–18 months. Young are born live, remain hidden under the mother for several hours or days, and then disperse.

**Orange-Headed Roach (*Eublaberus prosticus*)**
This is a larger species 1.5-1.9 inch (38–48 mm) that is a prolific breeder. Sexual maturity occurs between 3-5 months. These roaches can live up to 24 months. Due to aggression, house these insects in as large an enclosure as possible. If not provided with adequate space, moisture, and high-protein food, orange-headed roaches become cannibalistic, biting the wings of other adults and eating freshly-shed adults or nymphs. Ensure that plenty of water is available at all times in the form of chopped fruits or vegetables. These roaches are winged, but flightless and incapable of climbing glass or smooth surfaces.

Depending on the production needs, colonies may be established in containers ranging from 10 gallon (38 L) aquariums to plastic containers [30 gallons (114 L) or larger]. Cardboard egg crates may be stacked in multiple layers for furnishings. No substrate is necessary, and in fact, may make collection more difficult. The roaches will make refugia out of the multiple layers of egg crate.

Temperatures should be kept at 80–90 F (27–32 C). All three roach species discussed can handle temperatures lower than this, however reproduction declines dramatically at temperatures lower than 80 F (27 C), or may cease completely.

Finely ground premium dry dog food or crushed high-quality tropical fish flakes should be offered at all times in a shallow dish. This part of the diet should be kept dry at all times to

26 Available from Pro Exotics

prevent potentially harmful mold growth. As a source of moisture and vitamins, a variety of chopped vegetables should be offered at least three times per week. Remove unconsumed vegetables after 24 hours to prevent mold in the colony.

Smaller roach colonies should be cleaned weekly, and more frequently for larger ones. Due to their usually dry fecal pellets, sweeping out the enclosure is often sufficient, although disinfecting the container should be undertaken every 1–3 months depending on the number of roaches in a colony. Egg crates should also be replaced as they become coated in feces.

**Springtails**

Springtails are essential to culture when rearing small species or recently metamorphosed amphibians. Springtails (*Collembola* spp.) are white or grey-colored hexapods, small insects that live in the soil and eat fungi, bacteria, and plant materials. Some species are carnivorous, eating nematodes and other springtails. These tiny insects are often seen in the soil of potted plants. Starter cultures can be purchased commercially.[27] Springtails can be cultured in a plastic sweater box with a tight-fitting lid. Add the starter culture to a 1.5 inch (4 cm) layer of commercially-prepared, sterilized potting soil (free of added fertilizers). Mix soil with sphagnum moss and keep it wet. Sprinkle brewer's yeast and a little fish flake food on the top of the soil. Place clumps of tree fern or cork bark on top of the soil (Figure 24). The springtails will colonize the pieces of bark, which can then be rotated through the animal enclosures and back into the springtail colonies. Springtails can be kept at a range of temperatures and usually will do fine at regular room temperatures. For more information about springtails refer to Emmer (1993).

**Figure 24.** A springtail culture in a plastic shoebox. Cork bark pieces or fern fiber can be used to inoculate amphibian enclosures with springtails by transferring pieces of bark into a terrarium. (Photo: J. Pramuk)

**Confused Flour Beetles**

Confused flour beetles (*Tribolium* spp.) can be fed as larvae or adults to amphibians and are very easy to rear. Starter cultures can be obtained commercially.[28] To set up a culture of beetles, fill a plastic shoe or sweater box halfway with unbleached, enriched flour. Introduce a starter culture of beetles and larvae. Within a month or two, larvae and beetles can be harvested by using a screen or flour sifter. Offer the beetles and larvae on a shallow dish on the floor of the amphibian tank. Not all animals will eat beetles; however, they are a good alternative to pinhead crickets and fruit flies for smaller amphibians.

**Earthworms and White (Grindal) Worms**

Earthworms and white worms are a rich source of lipids and protein and are a great food item for underweight amphibians. Earthworms have one of the best calcium: phosphorous ratios of any feeder invertebrate. Earthworms are readily available from any bait supplier in a variety of sizes. A relative of earthworms, white worm starter cultures can be obtained commercially.[29] To set up white worms, use the substrate and enclosure described above for springtail cultures. White worms should be kept cooler than most cultures (approximately 60–68 F/15–20 C). To culture white worms, place a slice of wheat bread on top of the soil after adding worms. Set a small piece of plate glass or acrylic on top of the bread, allowing

27 Available from Ed's Fly Meat or L.F.S. Cultures
28 Available from Ed's Fly Meat or L.F.S. Cultures
29 Available from AquaBid.com and L.F.S. Cultures

worms to congregate between the bread and glass. Worms can be rinsed or scooped off onto an amphibian-feeding dish. If the room where the culture is housed is warm, consider acquiring an electronic wine cooler, which can be maintained at 60–68 F (15–20 C).

**Blackworms**
Blackworms (*Lumbriculus variegatus*) are a convenient, nutritional food source to feed salamanders and smaller frogs. They can be ordered from several suppliers or acquired at local pet stores. The worms can be maintained in a plastic sweater or shoebox with a secure lid and covered with just enough water to submerge the worms and stored within a refrigerator. The worms need to be rinsed with cold, fresh water everyday. Dead worms will float out with the effluent water and can be rinsed down the drain. The live worms will clump together and are easy to harvest. Worms can be placed on a shallow feeding dish for terrestrial animals.

**Fish and Rodents**
Fish such as minnows, goldfish, or rosy reds can be fed to larger species of amphibians.[30] Feeding frozen, thawed fish to amphibians exclusively can lead to thiamine (Vitamin $B_1$) deficiency, because both freezing and long-term storage (in fish containing thiaminase enzyme) both can destroy thiamine outright. It is better to vary the diet with live fish or avoid feeding frozen fish altogether.

Rodents include the following sized mice or rats: neonates (pinkies), fuzzies, hoppers, and adults. They can be fed to larger amphibians, although they should be offered sparingly due to their very high fat content. Neonates, in part because of the mother's milk in their stomachs, are particularly fatty and cholesterol-laden. Overfeeding these items to amphibians can lead to obesity and related health problems such as fatty liver, kidney failure, gout, and lipidosis. However, if an animal is underweight, offering neonate mice or wax moth larvae are effective for rapidly boosting weight. Rodents should be offered dead rather than alive to reduce chances of amphibians being injured.

## NATURAL HISTORY AND BEHAVIOR

Behavioral considerations cannot be understated as promoting normal behavior in captive animals ultimately leads to greater longevity and breeding. Replicating conditions that mimic natural activities and reproductive strategies and providing natural food items of the species are important and can be improved through enrichment techniques. Behavioral enrichment is a dynamic process that alters an animal's environment. Hypothetically, by providing stimuli to offer choices and to encourage natural behaviors, their welfare is enhanced. Although amphibian brains lack the highly developed cerebrum that is common to mammals, amphibians similarly have natural behaviors that can be encouraged in captivity and that are thought to increase their quality of life and overall health. Primarily, behavioral enrichment for amphibians focuses on methods that replicate the natural environment as much as possible, inducing normal behaviors.

One of the most important natural behaviors for all animals is feeding and the feeding response. One form of enrichment is to offer a wide variety of prey items, providing a broader nutritional base and creating a more complex captive existence. Additionally, not only the variety of food but also the way food is offered may promote natural behavior. While food items can be hidden or scattered throughout enclosures to encourage natural foraging and hunting behaviors, this should be done during times of the day when the amphibians are active, otherwise, prey may be able to evade predation by utilizing hiding spaces. Most

30 Such as cryptobranchid salamanders, Surinam toads (*Pipa* spp.), horned frogs (*Ceratophrys* spp.), bullfrog species, and large toads (*Rhinella* spp. or *Rhaebo* spp.), etc. Use forceps for feeding horned frogs and African bullfrogs to avoid being bitten by their odontoids (fang-like projections on the lower jaw).

amphibians are nocturnal, unfortunately making them difficult to exhibit, feed, and monitor during normal daylight hours. Reverse lighting can be employed using light timers, although it can be difficult to force the biological clock of an amphibian to mirror that of diurnal hominids. It is important to keep in mind "what," "how," and "when" we feed different species and keep in mind the difference between "keeper comforts" and "critter comforts." Cricket and fruit fly feeders can be created to release prey items into an enclosure slowly. These feeders are most useful for ant feeders such as dendrobatids and microhylids, which often wait patiently by ant mounds in the wild in an ambush strategy to catch their food. Another form of behavioral enrichment is training amphibians to "station" (coming to a designated location), enabling veterinarian and keeper staff to weigh or examine the animals without being handled, thus lessening potential for trauma and stress. Although this may sound like an impossible task for an animal as small as an amphibian, through hard work, it may be accomplished: for example, frogs could be trained to go to a weigh station at the sound of a clicker or salamanders could be conditioned to respond to the sound of tapping metal forceps to be fed.

The size and layout of enclosures, as well as social grouping composition, can affect behavior. Large or active species, such as some tree frogs (hylids) and African goliath frogs (*Conraua* spp.) require a lot of space. Enclosures that are too small can lead to abraded rostrums, other trauma, and even death. Live plants, cork tubes, and other "furnishings" serve as good perching and hiding areas that not only provide a sense of security for the animals and potential egg deposition sites, but also a way to prevent long jumps into the sides of the enclosure. PVC tubes partially buried make very secure hiding spots for ranids and some salamanders. In addition, substrates may be utilized as vehicles for stimulating natural behavior. Fossorial species such as caecilians and the Mexican burrowing toad (*Rhinophrynus dorsalis*) are far more at home in several inches of damp peat moss than on paper towels. Many amphibians are territorial in the wild and in captive situations can form dominance hierarchies. For example, male dart frogs and *Mantella* species will set up territories and combat for females and, even though dart frogs are usually solitary creatures, both female and male dendrobatids will become territorial during breeding season. Careful observation of collection animals aids in determination of sex ratios and for monitoring combat levels within enclosures. Plethodontid salamanders are highly olfactory and use pheromones to mark territories and attract females. Enclosures with residual pheromones from an earlier inhabitant might cause stress to a newly introduced animal. An animal's temperament should be considered, as well. For example, ambush predators such as African bullfrogs (*Pyxicephalus* spp.) and horned frogs (*Ceratophrys* spp.) cannot be part of a multi-specimen enclosure, because they will consume anything that fits into the gape of their mouths, including conspecifics (cannibalism).

For the sake of natural history accuracy it is recommended that multiple species be exhibited together only if they coexist in nature. Nothing completes these types of enclosures better than living plants that the species may encounter in nature. When geographically correct plant species are not available, substitute others that fulfill the need, especially in off-exhibit areas. This ultimately leads to more content and healthy amphibians.

## Amphibian Breeding

Amphibian reproductive cycles are closely linked to their physical and biological environments. Many effective breeding programs manipulate environmental cues such as temperature, humidity, and photoperiod. Altering these factors on an annual cycle that mimics the species' natural latitude is a good starting point for most amphibians.

### Cycling

In nature, amphibian breeding events vary by species but often follow some seasonal environmental changes (rainy/dry or cold/warm), thus simulated cycling, or artificial physical

conditioning, may be necessary to induce breeding in captivity. Environmental stimuli produce physiological changes needed to induce viable egg development and laying or sperm production. It is very important to research the individual species and learn as much as possible about natural breeding seasons and cycling. For temperate amphibians, a period of one to four months of lowered temperatures followed by a gradual warming period can induce breeding in many species. For this to occur, animals should undergo a period of fasting prior to being cooled down. At the end of the cooling period, the photoperiod and the temperature should be increased gradually. Environmental temperatures can be lowered to a minimum of 50 F (10 C) for many species from the temperate zone. Most animals are not completely dormant under these conditions, and remain at least slightly active, requiring clean water every day (i.e., animals are in a state of *brumation*). Comparatively, *hibernation* is a state of complete torpor, in which an amphibian will dramatically reduce its metabolism and food intake although it might still drink. As both are responses to cool temperatures, many amphibian culturists use wine coolers or modified refrigerators to simulate winter conditions for brumation of their animals. Make sure that sufficient water or moisture is provided during this period, as animals continue to require it.

In tropical regions of the world, breeding condition is often initiated by a rainy season following a brief drier period. Misting systems[31] attached to a timer can replicate rainy conditions, while synchronization to local rain events cue an animal's instinctual reproductive response to barometric changes. Temperature changes also can play a role in tropical species reproduction.

**Assisted Reproduction**
If breeding cannot be achieved through cycling methods, another widely used method to induce reproduction is to administer exogenous hormones (e.g., human chorionic gonadotropin or synthetic analogues of luteinizing hormone-releasing hormone injections). Assisted reproduction is covered in Chapter 2.

**Eggs**
Eggs should be treated with utmost care. All amphibian eggs possess layers of semi-permeable membranes that surround the ovum. Because they lack a hardened shell, they are essentially part of their surrounding aquatic environment. Even direct-developing eggs with relatively tough outer capsules uptake water from the damp substrate and should be kept moist at all times. If at all possible, avoid the temptation to move egg masses until the larvae hatch. Instances where this may not be possible include environments where the eggs can be consumed by other frogs or animals in an enclosure. Small, wet dip nets or spoons work well for transferring egg masses. Small tadpoles can be transferred with a turkey baster.

Eggs may be laid in water in large clumps, strings, or in small parcels at different sites. Many salamanders lay their eggs attached to sticks or vegetation in the water. There is much interspecific variation in the form and number of eggs laid, and the physiochemical properties of the eggs vary according to where they develop in the environment. See Duellman and Trueb (1994) for a thorough review.

Amphibian eggs have an animal and a vegetal pole (Figure 25A). In most cases, eggs of species whose embryos are exposed to sunlight are pigmented with melanin over the animal hemisphere. In contrast, most eggs that undergo development in concealed sites lack pigment and are light-sensitive (e.g., mantellas), as are the eggs laid on the undersides of leaves (e.g., those of *Phyllomedusa, Afrixalus,* and *Hyperolius*).[32] Yolks may be creamy-yellow,

31 Such as ProMist® products
32 These eggs should be shielded from light and if relocated, set in darkened areas or containers. Avoid flash photography if these eggs are valuable.

pale-grayish yellow, or in cases where the eggs are laid on leaves, pale green. Within several hours to a few days, early signs of development should be apparent, such as a clearly-defined yolk plug (Figure 25B) and elongation of the embryo (Figure 25C). If the entire clutch or an individual egg becomes fuzzy, these eggs are bad and should be discarded. This likely is the result of fungal grown on eggs that were not fertilized by a male. This could be caused by several factors including stressed animals (overcrowding), insufficient number of males to incite territorial behavior, poor nutrition, improper environmental conditions, or young inexperienced males.

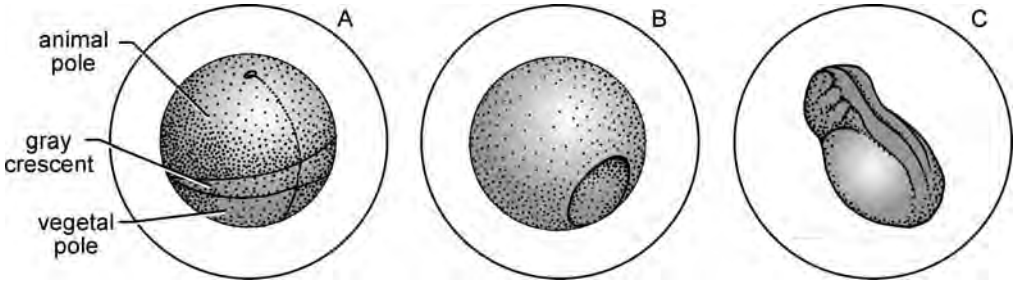



**Figure 25.** Amphibian eggs and an embryo in early stages of development. Within the first few days or even hours, signs of development should be visible. Earlier signs include clearly defined animal and vegetal poles (A), gastrulation (B) and (C) elongation of the embryo and neural fold development (the neural folds will become the spinal cord of the larva). Moldy (fuzzy) eggs will not develop and should be discarded. (Drawings: J. Pramuk)

The capsules of eggs laid in water will immediately swell by the uptake of water. Oxygenation of eggs is critical to their development and there is a continual increase in oxygen consumption throughout development. It is important to provide sufficient oxygenation, especially if there is a large clutch of eggs in a single enclosure. Amphibian embryos and larvae generally excrete nitrogenous wastes as the most toxic form ammonia. Additionally, amphibian embryos will develop normally only within specific limits of salinity and pH. Generally, development will occur faster in warmer environments but will plateau at a certain point (the ideal temperature). Eventually development slows as temperatures become too warm. Ensure the health of developing eggs and embryos by performing frequent water changes and water quality tests. Research on collection species may be necessary to ensure that setup is optimized for the health of specific eggs and larvae.

**Larvae Rearing**

It is important that background research is conducted on collection species before attempting to breed and rear them. Survival of most free-living amphibian larvae (tadpoles) is density-dependent. Although most tadpoles primarily consume plant material, many are omnivorous and some species are cannibalistic.[33] Larval mouthparts and digestive systems are adapted for specific diets and therefore a plant eater for example, probably will not survive on a diet of animal matter. Plant eaters have a longer digestive tract, which enables them to break down cellulose effectively.

33 Larval cannibalism may require some species [e.g., green and black poison dart frog (*Dendrobates auratus*)] to be held individually.

Water quality is extremely important for amphibian larvae. Water should be purified or, at the minimum, de-chlorinated if the municipal water source is relatively clean (see Water section above). Some species of frogs are adapted to living in tannic environments, such as in puddles of water on the floor of a tropical rainforest. These species can be reared in dilute tadpole "tea" that is brewed to mimic these tannic conditions and are thought to have natural antibacterial properties to prevent the water from becoming fouled (see sidebar for recipe).

**Tadpole Tea**
(Pramuk and Hiler, 1998)
1 oz. Alder cones (Alnus spp.) (Figure 24)
1 oz. German peat moss
2 quarts rain water

Add all three ingredients to a medium saucepan and simmer for about twenty minutes. Let cool. Add 0.5 cup of "tea" to 5 gallons of water.

Yield: 40 gallons of tadpole water.

Another trick adopted more recently by dart frog breeders is the use of Indian almond (*Terminalia catappa*) leaves in tadpole culture (Figure 26).[34] Breeders of Siamese fighting fish (*Betta)* have long used these leaves to reduce bacterial load in the water. Indian almond leaves also are attractive and can be used on the floor of a tropical frog enclosure either crushed or whole to provide antimicrobial tannins to the enclosure. These have been particularly effective with mossy frogs (*Theloderma corticale*).

**Figure 26.** Left, Indian almond leaves and right, alder cones. Both of these plant products can be used to increase tannins in tadpole water or in the substrate of an enclosure to limit bacterial growth and mimic tannic conditions seen in tropical forests. Dendrobatid tadpoles in particular do well when reared in tannic water. (Photo: J. Pramuk)

Water cleanliness can be maintained via mechanical and/or biological filtration, frequent partial water changes, or both. This is directly correlated with container size and volume.

Water-changes should utilize water of the same temperature as found in the tadpoles' enclosure. Tadpole enclosures can range from an aquarium with a filter for an entire clutch of offspring, to individual plastic shoebox containers (Figure 27), or deli cups used to house individual larvae. Tadpoles can also be held in a partitioned plastic tray (buttoner organizer) with the bottom replaced with screen (glued into place with silicone or a hot glue gun). Sheets of perforated PVC available from hardware stores can also be used to construct partitioned tadpole trays (Figure 28). These trays serve as a sieve that can separate the tadpoles from one another, but can allow them to be held in a common filtered body of water. This set-up offers the advantage of reducing cleaning duty, but risks sharing pathogens across the whole clutch (i.e., if one gets sick, the whole group is exposed).

34 Leaves can be obtained through dart frog breeders or in bulk from commercial vendors like AquaBid.com


**Figure 27.** Plastic shoebox-type containers are perfect for raising small groups of dendrobatid, centrolenid, or hylid larvae. These containers are not connected to filters and the water therefore will need to be changed at least partially on a regular basis. (Photo: R. Gagliardo)

**Figure 28.** A tadpole tray constructed of perforated sheets of PVC. The tray is sitting in an aquarium that is connected to an aquarium filter. While easy to maintain larger numbers of tadpoles separately, the re-circulating water could allow one diseased tadpole to infect the entire system. (Photo: J. Pramuk)

Regardless of whether the tadpole aquarium has a filter, regular water quality tests need to be performed and water will need to be changed periodically to reduce waste buildup. It is recommended that partial water changes are performed whenever the water is slightly cloudy, fouled, tadpoles are lingering near the surface, and/or waste is settling at the bottom of the tank. Replace approximately half to one-third of the water per cleaning event. If a filter is used, ensure that the current is not strong enough to draw the tadpoles up into the filter. A filter sponge can be formed around the influent end of the filter to slow intake and to prevent tadpoles from being pulled into the motor and killed. Once the hind limbs are well developed and there is evidence of forelimb development, provide a way for metamorphosing froglets to climb onto land, such as by reducing water volume and tilting the tank or providing a sloped substrate so that froglets can crawl onto a gradually exposed surface. Not providing a land surface for froglets or newly metamorphosed salamanders may result in drowning.

**Larval Foods**

Improper or insufficient tadpole nutrition can lead to metabolic and developmental problems such as spindly leg syndrome, which leads to permanently deformed and often crippled adults. Too much food at one time can foul the water and kill larvae. Research the feeding method employed and natural diet for the targeted species in advance.

Appropriate larval food includes the use of:

- Tetramin® tropical fish food flakes and tablets for dart frog tadpoles and those of many other species. Break tablets into quarters or smaller chunks depending on the portion tadpoles can eat within approximately ten minutes. Larger food items can be ground with an inexpensive coffee grinder.

- Sera Micron® and/or finely ground spirulina (blue green algae) for filter feeders such as some hylid tadpoles.
- Dark leafy greens (e.g., kale or mustard, but excluding spinach) frozen and thawed, or boiled breaks down cells and makes them easier for tadpoles to digest. Can also be used in combination with Sera Micron®.
- Sera Micron® smeared onto a feeding plate (i.e., microscope slide, Petri dish, rocks, or other inert material) allowing it to dry, and placing it at the bottom of the tank for grazing species (Figure 29).
- Mazuri® Amphibian and Carnivorous Reptile Gel is a nutritionally-complete gel diet that is prepared from a powder. Some amphibian tadpoles will consume the gel, but use sparingly as it fouls the water.

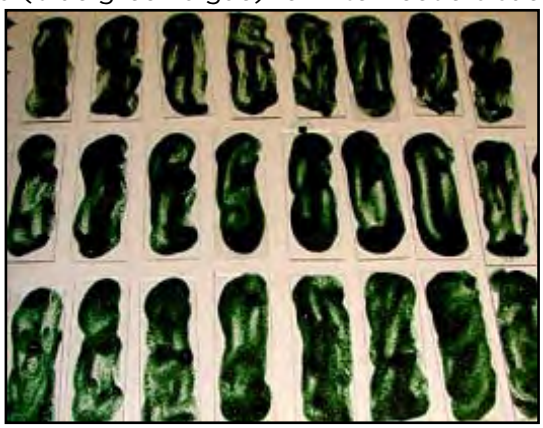


**Figure 29.** Feeding plates made from Sera Micron® paste smeared onto microscope slides and allowed to air dry prior to feeding. (Photo: R. Gagliardo)

- Rangen, Inc. Salmon Diet: Used with great success for feeding axolotls (*Ambystoma mexicanum*). Larvae are fed from 4 cm to adult.
- Hikari® Algae Wafers are a nutritionally-balanced algae based product that sinks in water. These have been used for bottom feeding *Atelopus* spp. and *Rana pretiosa* tadpoles.
- Although extremely labor-intensive, diatoms (green-brown algae) have been used as a tadpole food. A protocol for rearing diatoms is presented in Poole (2006).
- Homemade *Zippy Flakes* tadpole food is an excellent larval amphibian food (see sidebar for recipe).

**Zippy Flakes Tadpole Food**
(K. Zippel)

Mix the following ingredients:
16 g Sera Micron® powder
8 g Klamath Lake blue-green algae (cyanobacteria)
2 g Reptile/amphibian powdered vitamins such as Reptocal® mixed 1:1 with calcium carbonate.

Add water slowly until powder just forms a thick paste. Spread onto a sheet of glass or flat plastic dish. Dehydrate in refrigerator for two days. Scrape off flakes with a knife into an airtight storage container.

## VETERINARY MEDICINE

### Quarantine

A quarantine period should provide sufficient time for any health-related issues or symptoms to appear that are not related to the stresses of transport. It is recommended that new animals coming from outside the collection should be quarantined for a 60 days, with a minimum of at least 30 days. Ideally, amphibian quarantine should be located in a building separate from the rest of the collection, or at the very minimum, in a dedicated room. Quarantine should be an "all in, all out" procedure. Adding newly acquired specimens to a quarantine room effectively resets the clock for the quarantine period. New acquisitions may appear healthy and exhibit normal behaviors, but may be harboring fugitive pathogens to which other animals in the collection could be naïve and susceptible. Moreover, undergoing transport is stressful to most animals, leading to dehydration, overheating, starvation, stress from cage mates, or physical trauma to skin or internal organs. These stresses may depress the immunity of specimens and make them more susceptible to infection. Ideally, refrain from handling animals for the first few weeks when they arrive to quarantine. Minimizing the amount of contact to that which is absolutely necessary provides animals with a much-

needed period for a relatively stress-free acclimation. Collect and analyze fecal samples during this period. Given the ubiquitous nature of *Bd, c*onsider mandatory testing for this pathogen on incoming animals and prophylactic treatment after consultation with a veterinarian (Nichols and Lamirande, 2000). For more information on quarantine recommendations, see Chapter 4 and Pessier and Mendelson, 2010.

Modified plastic storage containers (Figure 8) or small plastic aquaria work well for temporary quarantine enclosures.[35] Paper towels can be used as a relatively sterile and disposable substrate; this will allow quick visual examination of the isolated animals as well as ease of fecal collections. The substrate should be changed daily and retain enough water that the enclosure remains sufficiently moist between cleanings. Paper towels can dry out alarmingly quickly in low humidity environments so check several times throughout the day to ensure that sufficient water is present. Enclosures can be partially covered in plastic food wrap to increase humidity. Throughout the quarantine period, handling of animals and human activity in the room should be kept to a minimum. Ideally, these animals should be serviced by personnel without other amphibians in their daily routine, or else these animals should be serviced at the end of the workday to reduce risk of cross-contamination.

**Parasites**

Wild-caught and even captive-born and -raised amphibians likely will harbor parasites. Often, animal dealers will hold captive-born animals in a cosmopolitan collection prior to shipment. This situation can lead to heavily infected animals that are shipped to unsuspecting recipients. Fecal samples should be examined throughout the duration of quarantine to assess the parasite load. Make sure that fecal samples are submitted to a veterinarian for examination. In collaboration with a veterinary professional, keepers should routinely check fecals as a matter of good husbandry practice. Direct and float fecal examinations can be performed in-house with a microscope and a few basic pieces of equipment. Both the presence of parasites and the seriousness of the parasite load can be determined using these types of exams. Depending on the philosophy of the attending veterinarian, up to three negative fecal exams might be required prior to releasing an animal from quarantine. Bloody stools also can be an indicator of parasite-infected animals.

Most wild amphibians harbor parasites that are symbiotic and do not harm their hosts; however, bringing animals with a parasite load into captivity can disrupt this equilibrium, and the parasites, if unchecked, may become fatal. Clearly, heavily parasitized animals likely will need some form of treatment in order to recover, but a healthy amphibian is not necessarily one that is 100% free of parasites. Effective anthelminthic and antiprotozoal treatments that can be prescribed by a veterinarian include levamisole, ivermectin, fenbendazole (Panacur®), praziquantel (Droncit®), pyrantel (Strongid-T®), and metronidazole (Flagyl®), or a combination drug such as Drontal Plus® (praziquantel/pyrantel pamoate/febantel). Ivermectin is not recommended as a first choice of treatments as it may cause serious side effects such as paralysis and even death (J. Pramuk, pers. obs.). See Chapter 4 and Wright and Whitaker (2001) for more information on treating amphibian parasites.

**Biosecurity**

Biosecurity involves three equally important aspects: 1) safety of the humans and scientists in an area; 2) decontamination/disinfection of field equipment (especially boots and nets) to prevent spread of a possible infectious agent to other sites and other animal populations; and 3) careful quarantining (isolation) of live, sick animals from all other populations in the field and in laboratory animal colonies (USGS, 2007). Strict antiseptic and quarantine-like measures should be taken to keep collections free of amphibian diseases and to reduce the potential spread of pathogens. This is important for all animals in the collection regardless of

35 Such as Critter Keepers® and Small Pal Pens®

life stage, but is especially critical for animals in quarantine. Use a disinfectant such as household bleach (3–6% sodium hypochlorite) to a 10% dilution to clean tools, empty enclosures, etc. and make sure that all surfaces are rinsed thoroughly and are free of chemical residue. Powder-free rubber, vinyl, or latex gloves should be worn when handling all amphibians and need to be changed between enclosures. Gloves should be moistened with a spray bottle or other source of clean water before handling amphibians. Best practice for quarantine and isolation facilities is to have dedicated footwear[36]; footbaths may supplement this practice, but should not be considered a substitute. When footbaths are used, they should be located at the entry and exit of each room to reduce the risk of pathogens being transferred between rooms. Footbaths should be filled with an antiseptic such as 10% household bleach and should be replaced daily. Every effort to eliminate pest animals (e.g., cockroaches, flies, rodents, or feral geckos) from amphibian rooms should be made, as they can be carriers of disease. Ideally, staff should wear a dedicated outfit or at least a dedicated lab coat for each amphibian room, which is washed daily. See Chapters 3 and 4 for more information about hygiene and biosecurity.

**Amphibian Diseases and Treatment**

There is no substitute for careful observation, so monitor animals closely everyday. These baseline data will provide invaluable information for evaluating the health of animals. At the first sign of unusual behavior or symptoms, contact a veterinarian with amphibian expertise. Often, hesitating by a day (or even hours) to treat a sick amphibian will be too late for treatment.

*Bd* can be devastating to a captive collection and wipe out an entire collection in a matter of days. In some species however, symptoms may not be expressed and carriers of *Bd* can remain unidentified in a collection for years. If amphibians are suspected of being *Bd*-positive, the most commonly prescribed treatment is 0.01% Itraconazole (Sporonox®) soaks for ten minutes daily for ten days (Nichols and Lamirande, 2000). Some veterinarians recommend treating all incoming amphibians prophylactically, whereas others prefer testing for *Bd* and only treating if the animals are positive. For more information on controlling amphibian diseases in collections, refer to Chapter 3 and also see Pessier and Mendelson, 2010.

## CONCLUSIONS

This guide is provided as a starting point for general amphibian husbandry. There are many other good resources available including those additionally cited below. We wish you luck in your amphibian propagation endeavors. Your work may be the last hope for some species to avoid extinction.

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36 Plastic shoes or boots that can be disinfected work well as dedicated quarantine footwear.

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Learn more about this class at www.aza.org/prodev/.

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## ADDITIONAL INTERNET AND PRODUCT SUPPLIER RESOURCES

### Recommended Amphibian Natural History Websites

www.amphibiaweb.org
A useful website maintained by UC Berkeley on taxonomy of amphibians. Usually has the most current list of amphibian species.

www.research.amnh.org/herpetology/amphibia/index.php
The American Museum of Natural History's *Amphibian Species of the World*.

Unfortunately, the taxonomy of the two above websites is often in conflict; however, the ATAG uses the taxonomy presented by the *Amphibian Species of the World* website. There is useful information on both websites, regardless of disagreements on which is the most widely-accepted amphibian taxonomy.

### Recommended Amphibian Care Websites

www.amphibiancare.com
A great site for general amphibian husbandry.

www.caudata.org
A detailed and informative site for salamander husbandry.

### Product List and Supplier Contact Information

**Room Heating/Cooling Units**
Sunpentown International
(800) 330-0388
www.sunpentown.com/wa12poacwihe.html

**Lighting Supplies**
Bulbster: Eiko® M16 halogen lightbulbs
Bulbster.com
(888) 323-2852
www.bulbster.com/lightbulbs/eiko-extsu-49487-p-1573.html

USA Light: M16 track light fixtures
www.usalight.com

**Terrarium Supplies**
AquaBid.com: Aquarium supplies, including Indian almond leaves
www.aquabid.com

Autograph Foliages: Artificial plants
(216) 426-6151
3631 Perkins Avenue, Cleveland, OH 44114
www.autographfoliages.com

Twin Oaks/Glasscages.com: Custom enclosures
(615) 446-8877
www.glasscages.com

ExoTerra®, ZooMed®, and Kritter Keeper®: Enclosures available at pet supply dealers.

### Horticultural Supplies

Agristarts Inc. (I-IV): Tropical plant tissue culture liners (plugs)
(407) 889-8055
1728 Kelly Park Road, Apopka, FL 32712
www.agristarts.com

Black Jungle Terrarium Supply: LECA, coco fiber, aquarium furnishings, plants, ExoTerra® enclosures, and other frog culturing supplies
(800) 268-1813
370 Avenue A, Turners Falls, MA 01376
www.blackjungle.com

Calwest Orchid Supplies: Sphagnum moss, cork bark, and fern fiber
(800) 301-9009
11614 Sterling Avenue, Riverside, CA 92503
www.orchid-supplies.com

Casa Flora, Inc.: Tissue culture liners of many types, natives, tropical, and ferns
(972) 225-5210
P.O. Box 41140, Dallas, TX 75241
www.casaflora.com

Deroose Plants, Inc.: Bromeliads and other tropical plants
(407) 889-5228
4601 N. Rock Springs Road, Apopka, FL 32712
www.derooseplants.com

Discoveries in Gardening: Premium quality sphagnum moss
New Zealand
(866) 241 9653 (international call free)
www.discoveriesingardening.com

Hummert International: Horticultural supplies
(800) 325-3055
www.hummert.com

OFE International, Inc.: Moss and supplies for orchids and bromeliads
(305) 253-7080
P.O. Box 161081, Miami, FL 33116-1081
www.ofe-intl.com

Tropical Plant Products, Inc.: Live tropical plants and supplies
(407) 293-2451
www.tropicalplantproducts.com

Tropiflora: Live tropical plants
(800) 613-7520
3530 Tallevast Road, Sarasota, FL 34243-3890
www.tropiflora.com

**Larval Amphibian Foods**
*Ambystoma* Genetic Stock Center: Rangen, Inc's. salmon pellets for axolotls
(859) 323-5679
101 TH Morgan Building, Lexington, KY 40506-0225
www.ambystoma.org/AGSC/food.htm

Mazuri: Amphibian and Carnivorous Reptile Gel diet
www.mazuri.com

Sera-Micron: Powdered fry fish food (only available in small 0.6 oz jars)
(800) 659-1970
158 Keystone Dr., Montgomeryville, PA 18936
www.sera-usa.com

Also available from Josh's Frogs (see below for link) or Black Jungle Terrarium Supply (see above for link)

TetraMin® Tropical Tablets and flake foods can be obtained from many pet food suppliers

**Vitamin Supplements**
Arcata Pets: Reptocal®
(800) 822-9085
www.arcatapet.com

Drs. Foster and Smith: Reptocal® and many other terrarium supplies
(800) 381-7179
www.drsfosterandsmith.com

Guenter Enderle: Nekton®-Rep and Nekton®-MSA
(727) 741-3386
2340 State Rd., Clearwater, FL 33763
www.nekton.de

Josh's Frogs: Repashy® vitamins
www.joshsfrogs.com/repashy-calcium-plus-4-oz.html

Vitamin-B Complex and One a Day® Men's Vitamins available from any drugstore

**Insect Culture**
Above Average Amphibians: Isopods, springtails, and bean beetles for culturing
Michael Shrom, shrommj@ptd.net
24 East Chestnut St.
Ephrata, PA 17522

Armstrong's Crickets: Crickets, mealworms, wax worms, red worms, and nightcrawlers
(800) 345-8778
PO Box 125 West Monroe, LA 71294
www.armstrongcrickets.com

Aubuchon Hardware/Hardwarestore.com: 1 pint wide-mouth glass Ball® jars
www.hardwarestore.com

Bassett's Cricket Ranch, Inc.: Crickets and mealworms
(800) 634-2445
365 Mariposa, Visalia, CA 93292
www.bcrcricket.com

Berkshire Biological: Pea aphid cultures and isopods
264 Main Road
Westhampton, MA 01027
Phone: (413) 527-3932 FAX: (413) 529-9382
www.berkshirebiological.com/record0019.html

Bioquip: Invertebrate collection equipment (e.g., black lights, butterfly nets)
(310) 667-8800
2321 Gladwick Street, Rancho Dominguez, CA 90220
www.bioquip.com

The Bug Farm: Invertebrate cultures and information on live food culture
www.livefoodcultures.com

Carolina Biological Supply: Fruit fly cultures, prepared medium (Formula 4-24®—order by the case to receive a discount), jars, and miscellaneous supplies
(800) 334-5551
www.carolina.com

Ed's Fly Meat: Wingless fruit fly and springtail cultures, and deli cups
(877) 359-6328
www.edsflymeat.com

Josh's Frogs: Plastic deli cups (32 ounce and 16 ounce sizes with perforated lids), fruit flies, and terrarium supplies
www.joshsfrogs.com
(800) 691-8178

Sefar America: Fine-mesh polypropylene screen for permanently mounting in fruit fly jar lids
(800) 995-0531

Sun Organic Farm: Organic beans and seeds for bean beetle and aphid cultures
www.sunorganicfarm.com/

Fluker Farms: Crickets, fruit flies, and meal worms
(800) 735-8537
www.flukerfarms.com

Grubco: Crickets, fly larvae, mealworms, and wax worms
(800) 222-3563
P.O. Box 15001 Hamilton, OH 45015
www.grubco.com

L.F.S. Cultures: Springtails, microworms, tubifex worms, grindal worms, white worms, red worms, fruit flies, flour beetles, and mealworms
(662) 236-4687
P.O. Box 607 University, MS 38677
www.lfscultures.com

New York Worms: Crickets, earthworms, wax worms, butter worms, and fruit flies
(516) 759-3538
7 Germaine Street, Glen Cove, NY 11542
www.nyworms.com

Worm Man's Worm Farm: Butterworms, nightcrawlers, crickets, fruit flies, mealworms, Phoenix worms, roaches, soldier grubs, fly larvae, and wax worms
(732) 656-0369
PO Box 6947, Monroe Township, NJ 08831
www.wormman.com

**Plumbing**
Aquatic Eco-Systems: Water filtration, pumps, bulkhead fittings, RO systems, phosphate absorbing media, and glass drill bits
(407) 886-3939
www.aquaticeco.com

Ecologic Technologies, Inc.: Rainmaker® misting system supplies
(410) 431-7106
P.O. Box 1038, Pasadena, MD 21123-1038
www.cloudtops.com/misting_system_index.htm

Glass-Holes.com: Inexpensive diamond-tipped drill bits for drilling tanks
www.glass-holes.com

McMaster-Carr: Bulkhead fittings, ball valves, tubing, and miscellaneous plumbing
(330) 342-6100
www.mcmaster.com

North Coast Pets: Diamond-tipped glass drill bits and bulkhead fittings
(877) 231-7416
www.northcoastmarines.com

Pro-Products: Specialized habitat control products including misting systems and heat panels
36 Split Rock Road Mahopac, NY 10541
(845) 628-8960
www.pro-products.com

Spectrapure: RO systems and other filters
(800) 685-2783
2167 E. 5TH Street, Tempe, AZ 85281
www.spectrapure.com

U.S. Plastics: Plastics including perforated PVC sheets for false bottoms, bulkhead fittings, ball valves, tubing, and miscellaneous plumbing
1390 Neubrecht Rd., Lima, OH 45801-3196
(800) 809-4217
www.usplastic.com

**Miscellaneous**
Ben Meadows: Equipment including data loggers
(800) 241-6401
Janesville, WI 53547-5277
www.benmeadows.com

Forestry Supply: Equipment including data loggers
(800) 647-5368
205 West Rankin Street, Jackson, MS 39284-8397
www.forestry-suppliers.com

Logtag Corporation Dataloggers
www.microdaq.com/logtag/index.php

Precision Weighing Balances: Weight scales
(978) 521-7095
www.balances.com

*Chapter 2*

# Assisted Reproductive Technologies (ART) for Amphibians

Andy Kouba[1], Carrie Vance[1,2], Natalie Calatayud[1,2], Trish Rowlison[1,2], Cecilia Langhorne[1,2], Scott Willard[2]

[1]*Conservation and Research Department, Memphis Zoo, 2000 Prentiss Place Memphis, TN. 38112 USA*
akouba@memphiszoo.org
ckvance@memphiszoo.org

[2]*Biochemistry and Molecular Biology Department, Mississippi State University, Mississippi State, MS. 39762 USA*
swillard@BCH.msstate.edu
tmr207@msstate.edu
nec62@BCH.msstate.edu
cjl224@msstate.edu

## 1. INTRODUCTION

The following chapter is meant to be a living document that will be regularly updated on the Association of Zoos and Aquariums (AZA) and Memphis Zoo websites as more is learned about the use of assisted reproduction for amphibian breeding. Assisted reproductive technologies (ART) are applied at various levels of the reproductive cycle and include everything from hormone supplementation for maturation and release of gametes to artificial insemination or fertilization, and even embryo management. Currently, ART has been applied to only a few amphibian species so there is limited knowledge about what the most efficient and safe methodologies are to induce natural breeding or to conduct in vitro fertilization (IVF). Virtually nothing is known about the application of ART for urodeles and caecilians so the majority of content for this chapter is what we know about anurans, frogs and toads. The authors would like to stress the importance of attempting natural breeding before resorting to ART as a means of reproducing brood stock. The *Amphibian Husbandry Resource Guide* outlines practical improvements for nutrition, hibernation, lighting, and exhibit construction that may provide more natural environments conducive to reproduction. All these husbandry manual recommendations may correct and improve the breeding situation for difficult-to-reproduce species and should be exhausted prior to resorting to ART as a means of growing the captive population. However, if natural attempts at reproduction have been exhausted and nothing has been learned about the animals' reproductive biology, then some high-risk species may have declined to a point that is unrecoverable, or important founders have been lost. Thus, it is recommended that a small experimental population be set-aside at the onset of any captive breeding program so that more can be learned about each species' reproductive biology. By doing so, the parameters for successful ART can be implemented immediately rather than being developed on a final valuable group of animals.

**WHY USE ASSISTED REPRODUCTION TECHNOLOGIES (ART)?**

Some wild amphibian populations have declined to a point in which captive assurance colonies have become necessary to save the species from extinction. Critical to the species' long-term survival is maintaining and breeding the founder population; however, often very little is known regarding the environmental cues that stimulate reproduction. In this scenario, time is working against the biologist, veterinarian, or zookeeper and ART may become necessary to save the species.

Before initiating any attempts at assisted reproduction in a new species it is recommended to contact specialists in this field of investigation and seek assistance with the design and implementation of ART protocols for captive breeding (names and contact information are provided in the Resources section of this chapter). This chapter will cover ART topics such as: what hormones are available for captive breeding in amphibians and how to choose which are the most appropriate, as well as standard operating procedures for hormone safety, storage, dosage calculation, and administration for conducting IVF, if needed. As the authors maintain a database on how ART is being applied and used in various species, they welcome input and would offer support on the use of ART in amphibians. Of particular importance is sharing both negative and positive experiences with the amphibian community so that others can learn from and repeat trials that work and avoid adverse situations or wasting time with negative results.

Amphibians display a wide range of reproductive strategies compared to mammals. The three living orders of Amphibia use both external and internal fertilization mechanisms reflecting oviparous, ovoviviparous, and viviparous strategies (Duellman and Trueb, 1986). Typically, anurans are oviparous, salamanders and newts are ovoviviparous, and caecilians are viviparous, although there are some exceptions to these categorizations, especially in anurans. The wide range of reproductive modes among amphibians mean that the development of species-specific protocols for ART will be necessary as the application of what is known about aquatic-breeding frogs with external fertilization may not be entirely applicable when trying to reproduce internally fertilizing salamanders or caecilians. For more information on amphibian reproduction, the authors recommend the extensive reviews on amphibian reproduction found in Duellman and Trueb (1986), Salthe and Mecham (1974), Whitaker (2001), Norris and Lopez (2010), Ogielska (2009), and for understanding the development of ART for amphibians it is recommended to review Clulow et al. (1999), Kouba et al. (2009), Kouba and Vance (2009), Browne and Figiel (2011) and the resources portal located on the Amphibian Ark website www.amphibianark.org.

Oviparous - External fertilization and development.

Ovoviviparous - Internal fertilization with external development.

Viviparous - Internal fertilization with birth of live young.

When first establishing an amphibian captive assurance colony, it is critically important to record basic characteristics such as age of first reproduction, age of puberty (e.g., first appearance of nuptial pads in males), seasonality, environmental conditions that triggered a reproductive event, fecundity, internal or external fertilization, egg size, oviparity or ovoviviparity, parental care, etc. This information will have significant implications for captive husbandry and breeding. A database should be created at individual institutions to record this material over the long-term, and it should also be shared via public portals within the amphibian conservation community. Animal studbooks are also an excellent resource for posting additional information on a species' life history and the AZA community would benefit from additional studies evaluating the content stored in studbooks. The primary goal of any captive assurance colony for threatened species is to reproduce the animals for future reintroductions once the original threat is eliminated, old habitat is restored, or new habitat designated (Figure 1). In order to create a sustainable population to meet this goal, the

**Figure 1.** In 2006, more than 2,000 critically endangered Wyoming toad *[Anaxyrus* (=*Bufo) baxteri]* tadpoles produced by IVF were released into the wild, marking the first time ART was applied to conservation for amphibians. This 'proof of concept' was a conservation milestone, highlighting how useful this technology could be for reproducing and reintroducing other threatened species.

amphibians must be able to undergo gametogenesis, egg maturation, ovulation and spawning/spermiation under captive conditions.

Unfortunately, it is very common for captive amphibians to exhibit reproductive dysfunctions; thus, it becomes necessary to modify the environmental conditions and failing that, apply hormonal therapy to exert external control over reproductive events. The next few sections will give a brief synopsis of the amphibian reproductive and endocrine systems along with common reproductive dysfunctions observed in captivity. While not exhaustive, the remainder of this chapter will provide the reader with the necessary information to explore the reproductive biology of a new species and experiment with the development of ART for the species of interest.

## 2. AMPHIBIAN REPRODUCTIVE SYSTEM

The amphibian reproductive cycle is controlled by a cascade of hormones originating in the brain hypothalamus, working through the pituitary gland, and terminating at the gonads (ovary or testes). This reproductive cascade is called the hypothalamic-pituitary-gonadal (HPG) axis (Figure 2). There are several important hormones that control the HPG axis, beginning with gonadotropin releasing hormone (GnRH), which is produced in the hypothalamus. When first discovered, this hormone was originally called luteinizing-hormone releasing-hormone (LHRH) because of its luteinizing hormone (LH) releasing-activity on the pituitary. However, LHRH is rarely used in the literature anymore because this neuropeptide also stimulates the release of another important hormone called follicle-stimulating hormone (FSH) that is involved with gametogenesis (development of the sperm and egg). Chemical companies continue to market GnRH as LHRH even though the name changed decades ago, leading to some confusion for those wanting to learn more about its use and application for amphibian reproduction. Because most of the amphibian literature addressing captive breeding and reproduction uses the LHRH nomenclature, this acronym will be used in this chapter. However, it is important to understand how these terms are used interchangeably so that individuals researching this compound and its applications are aware of the abundant literature using a different name, GnRH.

Gonadotropin releasing hormone (GnRH) is another name for LHRH and more appropriately reflects its action on the release of both gonadotropins, FSH and LH.

The amphibian brain is essentially the director of the HPG axis and assimilates environmental and internal cues leading to the appropriate response of neuroendocrine signals in which the primary signal is LHRH (Figure 2). LHRH is a decapeptide, meaning it is comprised of ten amino acids, and is the smallest protein known to vertebrates. Its action is highly localized; once it enters circulation endogenous LHRH only lasts about 5-10 minutes before being degraded by proteolytic enzymes. For this reason, initial attempts to use LHRH as a therapy for reproductive disorders in fish and amphibians were not effective. Eventually, researchers were able to create synthetic LHRH analogs (LHRHa) by modifying specific portions of the peptide making it more resistant to degradation and enhancing receptor binding. Today, these analogs are widely used in fish and amphibian reproduction for commercial purposes, laboratory studies, and captive breeding. The most common LHRHa used by zoos for captive breeding is **des-Gly$^{10}$, D-Ala$^{6}$, LHRH ethylamide**[1]. In addition, there is another LHRHa product commonly used for fish, des-Ala, Gyc$^{6}$-10 analog[2], which has been used by a few captive breeding programs including the Wyoming toad recovery group at Saratoga National Fish Hatchery, WY.

1 Sigma-Aldrich®, catalog #L4513
2 Argent Labs, item #C-LHRH-AN-1mg

The two primary gonadotropins produced by the anterior pituitary that stimulate the gonads are LH and FSH (Figure 2). At the initial stage of a reproductive cycle, FSH induces the secretion of androgens (testosterone) in males and estrogens (e.g., estradiol) in females. These steroid hormones are the ultimate effectors of gonad development and are critical for spermatogenesis in the male and follicle recruitment, oocyte growth, and vitellogenin production from the liver (used for sequestering egg yolk) in females. Near the end of gamete development, secretion of LH by the pituitary causes a shift in the steroidogenic pathway of the gonad and stimulates the synthesis and secretion of progestin-like compounds in females that initiate final maturation of eggs or impact spermiation in males. For many temperate and alpine amphibian species, the process of hibernation is critical for this final maturation process. Typically, reproductive dysfunction occurs in captive amphibians when there is an inability to replicate correctly the animals' seasonal environmental cues required to complete gametogenesis. In this instance, hormonal intervention becomes necessary to complete the process of egg maturation, stimulate ovulation, and induce spawning in females or spermiation in males.

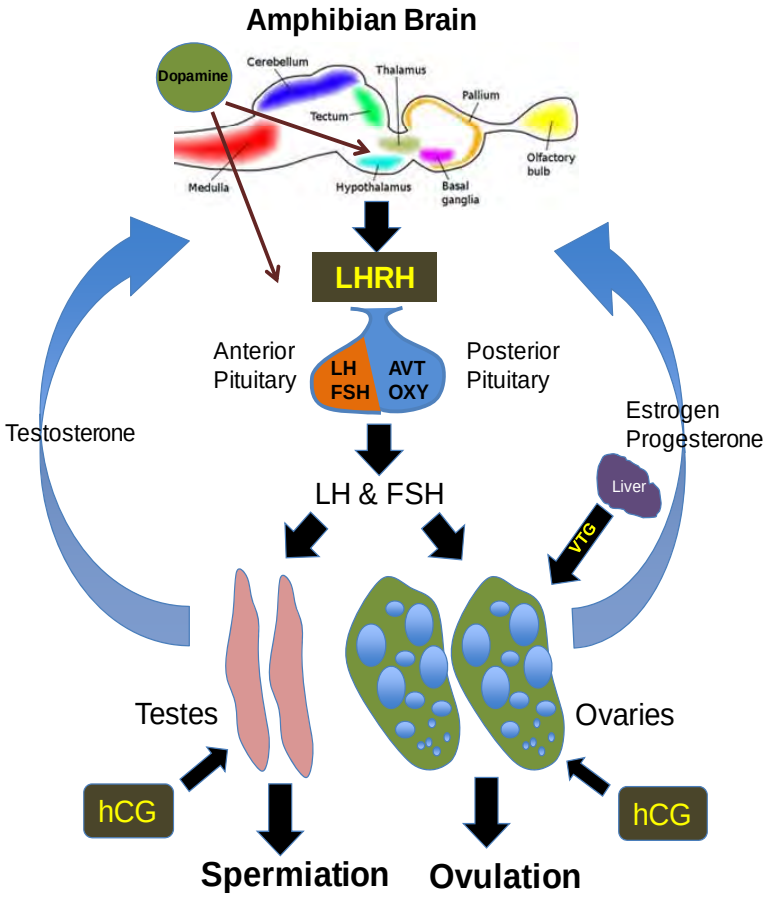


**Figure 2.** Hypothalamic-Pituitary-Gonadal (HPG) axis for amphibians. The brain releases LHRH, in response to environmental and internal cues, which then binds to receptors in the anterior pituitary. Receptor binding then leads to the release of FSH or LH depending on the stage of the reproductive cycle. FSH and LH stimulate steroidogenesis in the gonads which induces spermiation in the testes, follicle growth and ovulation in the ovaries, and vitellogenin (VTG) production by the liver. Steroids help regulate the process at the level of the brain and pituitary through feedback loops. Administration of LHRHa induces a natural cascade of endogenous hormones from the pituitary whereas administration of hCG acts directly at the level of the gonads to induce spermiation, follicle growth and ovulation. AVT=arginine vasotocin; OXY=oxytocin; LH=luteinizing hormone; FSH=follicle stimulating hormone; LHRH=luteinizing hormone releasing hormone, hCG= human chorionic gonadotropin.

**Reproductive Dysfunction in Captive Amphibians**

During their reproductive cycle, amphibians experience a variety of environmental cues that prepare them for a successful breeding event. In the wild, the HPG axis of the frog functions correctly and reproduction occurs successfully. Unfortunately, most amphibians reared or moved to captivity often show reproductive dysfunctions due to their captive conditions (Figure 3). Even those species that reproduce reliably year to year in captivity typically have reduced fecundity compared to their wild counterparts. These reproductive dysfunctions can occur in both sexes, with males showing reduced behaviors such as amplexus (Figure 4) and lack of calling, while females fail to ovulate or have reduced egg numbers and egg quality. These dysfunctions arise from three main causes: 1) poor nutrition; 2) missing environmental stimuli conducive for reproduction; and 3) stress associated with captivity. These three root causes, either alone or in combination, are responsible for the total or partial inhibition of reproduction currently observed in the majority of captive amphibian populations.

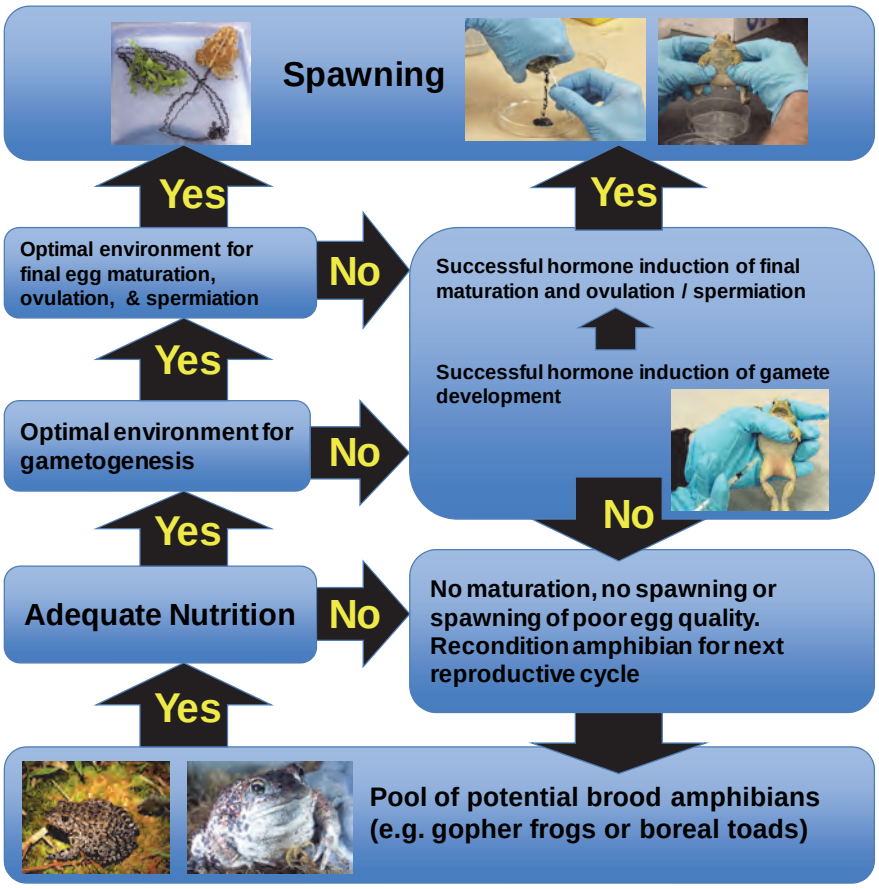


**Figure 3:** Decision tree for critical points in the amphibian reproductive cycle, with timing for hormone therapy intervention. The left side of the diagram is a complete and successful reproductive cycle where nutrition and environmental parameters are optimal, resulting in natural spawning. The right side of the diagram represents inhibition at various reproductive phases that require intervention by improving either the nutritional plane of the animal or intervening with hormone therapy to facilitate gametogenesis, final egg maturation, ovulation, and spawning (females) or spermiation (males). Hormone therapies may require the use of low-dose priming hormones and/or larger ovulatory dosages to induce ovulation and spawning. Even if spawning fails to occur following hormone therapy, eggs can manually be stripped from females and fertilized in-vitro with sperm collected from donor males. To date, all critically endangered gopher frogs *[Lithobates* (=*Rana) sevosus]* offspring have been produced by in-vitro fertilization (IVF) following egg stripping and sperm collection. Similarly, in the boreal toad, IVF has been used to produce thousands of fertilized eggs and tadpoles, which were subsequently released into the wild.

**Figure 4.** Amplexus behavior in a male boreal toad following a cocktail injection of LHRH + hCG.

The goal for each curator and keeper should be to minimize the negative impacts of each of these factors in order to optimize reproductive performance. Information contained in Chapter 1 of this husbandry manual outlines standard operating protocols for creating natural appearing exhibits that create various ecological microhabitat niches in three-dimensional space and can reduce the impact of the third cause of reproductive dysfunction, stress. It is well known that stress in amphibians increases cortisol levels that inhibit several endocrine pathways critical for gametogenesis and reproductive behavior, specifically the release of LHRH and arginine vasotocin (AVT) (Carr, 2011). Often space is a limiting factor and the challenges described above cannot be corrected sufficiently to entice natural behaviors (e.g., foraging, dispersal, or establishment of territory for grouped exhibits). One method to help overcome this space limitation is the creation of a few large breeding enclosures, off-exhibit, that could be used for numerous species with animals cycled out of the naturalistic enclosure depending on the timing of their seasonal breeding. The authors are unaware of detailed studies showing how stress levels fluctuate between these two types of enclosures with impacts on reproduction but such information would prove valuable.

The second cause of reproductive dysfunction is the absence of environmental stimuli permissive to reproduction, and is much more difficult to correct. Mimicking the complexities of the natural environment, from post-breeding, through hibernation, and culminating with locating or migration to appropriate spawning sites, is nearly impossible to replicate in captive situations. Moreover, many species are in captivity because the wild populations are threatened with extinction and virtually nothing is known regarding their reproductive ecology. Those programs that have had some success with replicating natural environments are familiar with the natural history of the animals in the wild. A critical component to any recovery program is to establish assurance colonies in the same country of origin, preferably in the same state or province (with similar elevation) to where the animal is found; thus, exposing the captive colony to the same environmental stimuli. If possible, outdoor enclosures (with hibernacula for temperate or alpine species) would provide the most appropriate natural conditions. The adult amphibian has evolved physiological mechanisms to detect environmental stimuli optimal for breeding and will either: 1) under optimal conditions carry out reproduction to successful spawning; 2) under sub-optimal conditions arrest gamete development and postpone reproduction until suitable conditions present themselves; or 3) under poor conditions terminate gamete development, resorb nutrient materials invested in the eggs, and return the gonad to a resting state. These three endocrine routes developed to ensure the survival of offspring and parent. Captive female amphibians, more often than males, experience reproductive dysfunctions associated with inadequate environmental stimuli.

The Wyoming toad recovery program has established breeding colonies near their native habitat which helps acclimate the captive animals to the same environmental conditions as their wild counterparts. Animals are placed into outdoor hibernacula that encourage natural reproductive behaviors and gametogenesis.

The last cause of reproductive dysfunction, inadequate nutrition, is a strong external factor impacting the HPG axis and usually results in the shut down of the reproductive system during vitellogenesis in females. The mass of fat bodies in amphibians is directly linked to reproductive success and provides the energy reserves for many physiological processes, including their survival during hibernation and continued gametogenesis. Removal of fat

bodies impairs gamete development in both male and female amphibians; these fat bodies serve as an immediate source of nutrients for gonadal activity, especially vitellogenesis, through transfer of essential lipids and proteins to pre-vitellogenic follicles (Rastogi et al., 2011). The first physiological process to shut down when an animal is under nutritional stress is the reproductive system, as it is a non-essential activity for the animal's immediate survival (*motto*: live to reproduce another day). In this instance, all manner of exogenous hormones can be given to the individual and it will not matter as the animal does not have any follicles recruited that can go through vitellogenesis. Observations from most captive amphibian populations that have been established for several years indicate that a proportion of animals might never respond to hormone treatments. This percentage loss of breeding animals could be due to nutritional reproductive failure. If an assurance colony has been established for a threatened species with the goal to reproduce the animals for long-term management and future reintroductions, then the animal's nutritional state must be a primary consideration. Any deficiencies early on in the animal's development can have long-term, chronic consequences. One problem zoological institutions have is finding enough of a varied diet to offer amphibians. A one-size fits-all diet of crickets has probably contributed to the widespread collapse of reproductive activity in captive amphibians, even with vitamin dusting and gut loading of the crickets. Invariably, species provided with a wider diet offering, such as red worms, beetles, ants, spiders, earthworms, and mealworms are healthier and less likely to exhibit pathological deficiencies. A good example is the high rate of squamous metaplasia seen in captive Wyoming toads *[Anaxyrus (=Bufo) baxteri]* due to vitamin A deficiency (Pessier et al., 2005; Densmore and Green, 2007). Wild toads consume mass quantities of ants and beetles (Baxter and Stone, 1985) that are high in β-carotene and retinol (Pennino et al., 1991) resulting in vitamin A values much higher than their captive counterparts. More studies are needed to understand the link between nutrition and reproduction in amphibians with corrective mechanisms (i.e., dietary protocols) for resuming natural reproductive processes so that the need for ART is reduced.

A varied diet will improve an animal's nutritional state and reproductive success. Possible diet may include: crickets, red worms, beetles, ants, spiders, earthworms, and mealworms.

Reproductive dysfunctions in female amphibians are more challenging to correct than for males due to the various problems with: 1) inhibition of vitellogenesis; 2) inhibition of egg maturation; and 3) inhibition of spawning. The first type of inhibition, block of vitellogenesis, is the most serious and means that the reproductive process did not even begin. Typically this is related to poor nutrition or environmental factors as previously described. The second inhibition, lack of egg maturation, is probably the most common and causes the post-vitellogenic eggs to degrade (atresia) and resorb. Occasionally, a small pool of the eggs will complete maturation leading to spawning of reduced egg numbers of poor quality (lower fertilization rates). The third dysfunction in females, inhibition of spawning, is the least serious complication. In this instance, the eggs have completed all phases of the reproductive cycle including ovulation, but are retained in the female's abdominal or ovarian cavity and never laid. This is probably the second most common problem with female amphibians and typically results in the eggs degrading and being reabsorbed. A large number of zoological institutions have contacted the principal author over the last decade indicating that genetically valuable females that appeared to be healthy were expiring and upon necropsy were full of eggs. Although widely accepted by veterinarians that egg-bound females can die from an inability to reabsorb their eggs, there are no published studies on dystocia (egg retention) in amphibians, yet it is well documented in reptiles. For those species that appear to be more susceptible to the adverse effects of egg retention, manual removal of the eggs by stripping them from the female can be accomplished following hormone stimulation (see section on Hormone Use for In Vitro Fertilization).

The principal male dysfunction observed in captivity is the absence of appropriate reproductive behaviors such as failure to perform advertisement calling and amplexus, even after artificial hibernation for temperate species. Once the male goes into amplexus a hormonal cascade is initiated that prepares the male for sperm release upon spawning by the female. Whereas, nutritional deficiencies can have profound effects on female reproduction, this is likely not the case with males; the predominant factor affecting their performance is probably inappropriate environmental cues conducive for reproductive behaviors.

## 3. HORMONE THERAPY FOR REPRODUCTIVE DYSFUCTION

Hormone therapies for assisted reproduction in amphibians can be broken-down into two categories, *first generation* and *second generation* effectors. Not only do these descriptions relate to the discovery time-line and use, but also the level at which the HPG axis is affected (Figure 2). First generation hormones include pituitary homogenates/extracts, purified pituitary gonadotropins (LH and FSH), or human chorionic gonadotropin (hCG) and act directly at the level of the gonads. Second generation effectors act at the level of the brain and include LHRHa and dopamine antagonists, which stimulate the gonads indirectly by acting at the pituitary to release the animal's own endogenous LH and FSH. Understanding the targeted action of these two drug classes can have profound effects on the response of individual species and their successful application. Inducing the animal's natural internal hormonal cascade with second generation hormones can have a stronger effect on specific reproductive behaviors, spermiation, and ovulation. However, species-specificity may occur such that the pituitary of the treated amphibian remains unresponsive to treatments. In these cases, hormonal treatments may be ineffective because LHRHa fails to induce a natural hormonal cascade of LH or FSH; whereas first generation drugs, which act directly on the gonads, do not depend on the functional specificity of the pituitary. For example, hCG is a better stimulator of sperm production for male Fowler toads [*Anaxyrus (=Bufo) fowleri]* than LHRHa but will not elicit male amplexus of females. Consequently, when trying to promote natural breeding, LHRHa is the more appropriate stimulator of correct reproductive behaviors for this species, while hCG has the more localized effect on the gonad and can be used for sperm collection and ART. Furthermore, in some cases dual application of LHRHa and hCG may promote both natural behaviors and spermiation. The rest of this section will provide detailed information on several hormones used to induce egg maturation, ovulation, spermiation, and amplexus (Figure 5).

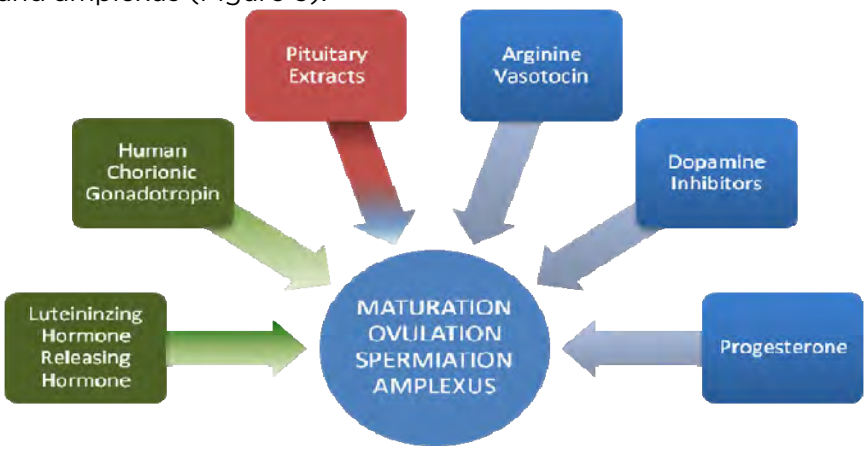


**Figure 5.** Various hormones used for inducing egg maturation, ovulation, spermiation, and amplexus for amphibians. Hormones in green blocks have a direct impact on the above-mentioned processes and are recommended for trial in endangered/threatened species. Pituitary homogenates/extracts (red block) are recommended for use only if all other avenues of investigation have failed to stimulate gamete production and spawning. The blue blocks are hormones that will not themselves induce spermiation, ovulation, or spawning, but play an important role in the success of reproduction and can affect hCG or LHRH effectiveness. The hormones in the blue blocks are still relatively experimental for most amphibian species and more data is needed on their use.

### 3.1 Pituitary Homogenates / Extracts

Pituitary homogenates are excised pituitary glands from a sacrificed individual that are subsequently crushed in a suitable medium to free up the hormones and are then administered to a recipient for purposes of inducing breeding or expressing gametes. Pituitary extracts go through an additional crude purification step to concentrate the hormones and remove most of the cellular debris. Pituitary homogenates are known stimulators of ovulation and spawning in female amphibians and to a lesser degree, spermiation in males (Subcommittee on Amphibian Standards, 1996). The use of pituitary homogenates to collect eggs and sperm has been in practice for nearly a century in several key laboratory species, and was the first method used for hormonal-induced collection and study of gametes from live animals.

There are several disadvantages to using pituitary glandular preparations from sacrificed animals. First, pituitary homogenate/extract may contain dangerous transmissible diseases that could be passed to the recipient. Given the global spread of pathogens, such as the amphibian chytrid fungus and ranavirus, the passage of these diseases to endangered or threatened species should be avoided (especially if the future goal is reintroduction). Second, animals must be sacrificed to perpetuate another animal's genetic line. Researchers and conservationists must weigh the ethical and acceptable risk of this sacrificial technique; for example, it may be ethically acceptable to sacrifice the invasive American bullfrog to save an endangered frog of the same genus from the brink of extinction if no other hormonal therapy worked. Third, the exact reproductive state and hormonal milieu of the donor animal(s) is typically not known and the active amount of gonadotropin available to the recipient is quite variable. Fourth, the homogenate/extract is comprised of other cellular debris and/or pituitary hormones that could have adverse effects on the recipient. Lastly, research indicates species-specificity response to the homogenates such that not all species react in the same way to pituitary preparations (Redshaw, 1972). Most commercial supply companies have discontinued the sale of frog pituitaries, possibly due to disease issues.[3] However, if required, there are papers discussing methods of collection and preparation should homogenates be needed in the future for conservation (Rugh, 1965; Subcommittee on Amphibian Standards, 1996). The use of homogenates is recommended only if various concentrations and drugs listed below have failed to produce a desired response.

### 3.2 Gonadotropins

The most common first generation gonadotropin used to stimulate spermiation in males or egg maturation, ovulation, and spawning in female amphibians is hCG. hCG was first isolated in human female urine and was widely used as a pregnancy test from the 1940s to the 1960s (Bellerby, 1934; Galli-Mainini, 1947a, b). After injection of human female urine into African clawed frogs *(Xenopus laevis)* or common toads *[Rhinella (=Bufo) arenarum]*, the presence of eggs or production of sperm was monitored in the female or male amphibian, respectively. This method became known as the "Bufo test". While injections of pregnant human female urine performed the same function in other mammals such as mice and rabbits, toads were less expensive to maintain in a hospital or clinic and did not require euthanasia to confirm ovulation. With the discovery of monoclonal antibodies and immunoassays in the 1970s, the modern home pregnancy test was developed, halting the worldwide distribution of amphibians for pregnancy testing.

hCG is produced by the chorionic membrane of the placenta and is the pregnancy recognition factor in humans, maintaining the production of progesterone by the ovary and sustaining pregnancy (Johnson and Everitt, 2007). The protein has LH-like activity and

3 The authors are currently unaware if a commercial vendor of frog pituitaries exists in the U.S.

therefore stimulates the gonads directly and activates steroid biosynthesis. This causes the release of sperm in males, or maturation, ovulation, and spawning in females. Because the hormone is of human origin, the protein does not have the same efficacy in amphibians as it does in mammals, resulting in the effective dose for amphibians being nearly 2000 times higher than that given to a mammal on a per weight basis (Kouba et al., 2009). Even though hCG shows reduced specificity and is required in larger amounts to be effective, it is widely used due to its standardized activity [International Units of Activity (IU)] that can be adjusted on a per weight basis, as well as its low cost and high availability in the world market. The hormone hCG can be readily purchased and ordered in different quantities, typically 2500, 5000, or 10,000 IU, depending on the amount needed to meet long-term breeding goals.[4]

Purified pituitary gonadotropins, FSH and LH, derived from human and other mammals have been tested in amphibians with little success. The lack of gonadal stimulation from these compounds is most likely due to the species-specificity and receptor recognition of the proteins. Unfortunately, there is limited commercial interest in overcoming the technological challenges of isolating and producing amphibian LH and FSH in mass amounts, thus there are no products available for purchase. Having such products would be of great benefit to reproductive physiologists studying and applying ART in captive amphibian populations. Benefits would include: reduced risk of disease transmission, accurate dosing, repeatability of treatments, stronger pharmacological efficacy at lower doses than hCG, less risk of immunological responses, and better efficiency than mammalian gonadotropins. Until the scientific community has purified amphibian LH, hCG will be the general gonadotropin of choice.

**3.3 Luteinizing Hormone Releasing Hormone**

The second generation hormone LHRH is a small decapeptide produced by the hypothalamus of the brain that stimulates the endogenous production and release of LH and FSH from the pituitary. There are several advantages of using LHRH over other hormone therapies because it acts at the level of the brain to stimulate the animal's own reproductive processes. In female amphibians, LHRH stimulates final egg maturation, ovulation, and spawning while in males it can increase sperm volume and concentration (Kouba et al., 2009). In addition to initiating the animal's natural reproductive processes and behaviors, other advantages of using LHRH instead of hCG are that the LHRH analog mentioned previously (des-Gly$^{10}$, D-Ala$^{6}$, LHRH) is generic and is applied to a broad range of species from fish to mammals, making it widely available commercially. It is imperative to note that finding the correct hormone concentration is necessary and will likely be very species-specific. A typical LHRH injection will induce an LH surge that can last 12-48 hours in amphibians. Second, because of the small size of the molecule (10 amino acids), it does not generate an immune response and repeated injections can be given without desensitization. However, it should be noted that anecdotal information suggests that repeated injections timed too frequently (i.e., days) may down-regulate or desensitize the receptors in the pituitary, resulting in loss of potency. Although there are more than 25 different LHRH compounds available for sale by Sigma-Aldrich® the most common analog used by the amphibian community is des-Gly$^{10}$, D-Ala$^{6}$, LHRH ethylamide (catalog #L4513). While limited comparative trials have been conducted, when other LHRH analogs have been tested against L4513, they have not performed as well in stimulating spermiation and ovulation in amphibians. Most of the information on the effectiveness of other analogs compared to L4513 comes from research on fish (Cabrita et al., 2009).

4 Sigma-Aldrich®, catalog #CG5 (5,000 IU) or catalog #C1063-10VL (2,500 IU); or through Animal Health International, Inc (formerly DVM Resources), trade name Chorulon®, catalog #IVA022219

### 3.4 Progesterone, Dopamine Antagonists, and Arginine Vasotocin

There are several important support hormones that should be mentioned which can increase the effectiveness of LHRH or hCG, but themselves do not stimulate spermiation, final egg maturation, ovulation, or spawning. For example, the steroid progesterone stimulates final maturation of amphibian eggs. If the reproductive dysfunction originates from lack of egg maturation, single injections of LHRH will not be sufficient to induce maturation and ovulation, culminating in spawning. Rather, a set of repeated injections of LHRH or hCG becomes necessary to complete maturation through a process called priming (see Section 8). However, these multiple injections might be circumvented through the use of the natural egg maturation promoting-factor, progesterone. There are several challenges to using progesterone, not the least of which is its lack of solubility in water-based mediums. Currently, the authors are investigating options for using progesterone to complete maturation of eggs in species that do not respond well to LHRH or hCG treatments alone.

Another important control mechanism of the HPG reproductive axis (Figure 2) is dopamine (DA). The neurotransmitter DA exerts an inhibitory effect on LHRH synthesis in the hypothalamus, down-regulates the LHRH receptor in the pituitary, and directly inhibits LH secretion from the amphibian pituitary (Sotowska-Brochocka et al., 1994). A common practice in fish aquaculture is to administer a combination of LHRH with a DA antagonist that blocks or removes the inhibitory action of DA, with the goal of making the LHRH much more effective in stimulating a strong LH surge. There are three main DA antagonists that have been successfully used in combination with LHRH in fish aquaculture: 1) metoclopramide, 2) pimozide, and 3) domperidone. There are two commercially available pellets that combine LHRH + a DA antagonist for administration to fish called Ovaprim and Ovopel, but to the authors' knowledge have not been tested on amphibians. A recent study in frogs showed that the combination of LHRH + metoclopramide was able to stimulate female northern leopard frogs *[Lithobates* (=*Rana) pipiens]* to spawn in captivity both during the natural breeding season and environmentally-conditioned out-of-season (Trudeau et al., 2010). The combined LHRH + DA antagonist did not work as well on animals collected out of the breeding season, because the artificial hibernation protocol was likely not optimal (Trudeau, V.L., pers. comm.). The paper's authors have named the technique "Amphiplex" and recommend anyone interested in this formulation contact the researchers involved with its design for advice and collaborations (see Resources section). To date, the "Amphiplex" method has been successfully used in three species of *Ranidae*, *Xenopus laevis*, and several Argentinian frogs (Trudeau, V.L., pers. comm.). Due to the potential side-effects inhibiting brain neurotransmitter activity can have if over-dosed with a DA, it is strongly recommended to contact the developers of this technique for advice before beginning any treatment. In 2009, the U.S. Food and Drug Administration (FDA) issued a black box warning regarding long-term or high-dose use of metoclopramide because of the risk of developing tardive dyskinesia that causes morbidity and movement problems in many human patients (Rao and Camilleri, 2010). The LHRH + DA antagonist combination has only been used on a few amphibian species to date, and as more trials are performed more will be learned about its potential use, side effects, and which species are responsive versus not responsive. For example, marine fish do not have a strong DA inhibitor system, so the addition of a DA antagonist has no additional benefit for egg maturation, ovulation, and spawning compared to LHRH alone (Cabrita et al., 2009).

Arginine vasotocin (AVT) is a neurohormone produced within the amphibian brain and released by the posterior pituitary (Figure 2). AVT acts locally within the brain to modulate social and reproductive behaviors and is known to increase sexual arousal in some male amphibians and induce certain behaviors, such as advertisement calling and amplexus (Propper and Dixon, 1997). The hormone has also been shown to induce ovulation and phonotaxis in some female amphibians (Rose and Moore, 2002). AVT acts primarily on the

central nervous system and has no direct link to the gonads. A possible disadvantage to using AVT is that it can cause an inhibitory effect on sperm release when given in combination with LHRH, such as in Günther's toadlet *(Pseudophryne guentheri)* (Silla, 2011). As a result, hCG has been suggested as a substitute for LHRH, when given in combination with AVT. Also, spacing out the hormone injections and administering LHRH first might allow the hormone time to stimulate gonadotropin secretion. hCG acts directly on the gonads, bypassing any inhibitory effect on the pituitary secretion of LH. The AVT system in urodeles is quite extensive compared to anurans and the use of this hormone for inducing breeding in salamanders may be valuable for species that are difficult to reproduce. AVT is also an anti-diuretic and can cause fluid retention. Therefore, it is advisable to supplement water via a moist sponge rather than in a pool, or by offering dry space in the holding tank. AVT can be purchased as [$Arg^8$]-vasotocin acetate salt[5]. It is recommended that AVT be reconstituted using a buffer such as phosphate buffered saline (PBS) or simplified amphibian ringer (SAR) solution, and then aliquot into smaller volumes, such as 100 µl, storing at -80 C. Once thawed, an aliquot can be stored at -20 C for approximately one month. AVT is typically measured in micrograms (µg) with doses of AVT generally ranging from 0.1-10.0 µg/g body weight. It is recommended that AVT be reconstituted in 200 µl buffer and injected intraperitoneally.

## 4. WORKING WITH HORMONES SAFELY

When working with reproductive hormones, following safe handling, storage, and disposal protocols is paramount to ensure personal and animal safety. These procedures not only protect the keeper, veterinarian, or investigator performing injections on amphibians, but also protect those around them, those that may enter or work in the space after them, and the animals with which they are working.

### 4.1 Safe Handling Practices

Some hormones purified from tissues or fluids of human origin (e.g., urine from pregnant women to collect hCG) should be considered a biohazard. While most suppliers have rigorous safety testing requirements, any hormones from human-sources should still be handled as though they are capable of transmitting infectious agents. To work safely with these chemicals, utilize the following recommendations:

- Before using any reproductive hormone, the operator should consult the Material Safety Data Sheet (MSDS) that came with the chemical. A typical MSDS contains all the information needed to assess health hazards, working conditions required, toxicology data, storage conditions, disposal, and cleanup in event of a spill. Prior to the chemical's use, the operator should have a clear understanding of any risks involved with handling.
- Gloves should be worn at all times, from the moment the chemical is removed from storage, throughout the entire process of handling the amphibian and its enclosure. The hormones can remain in the system of the frog for more than 48 hours (in the case of hCG) and can be passed to a handler through skin secretions or urine. Care should also be taken when handling any water the amphibian was sitting in, as the hormone or its metabolites could have been excreted into the tank. The amount of hCG given to an amphibian can be 2,000 times the effective dose given to a mammal on a per weight basis so its pharmacological actions on the handler can be quite strong if exposed (Kouba et al., 2009). The primary author is aware of one reported case in which a woman with frequent exposure to these hormones failed to follow these safety procedures and experienced a brief interruption of her regular menstrual

5 from Sigma-Aldrich®, catalog #V0130 or #V9879

cycle, underscoring the need for wearing gloves to protect the skin from absorbing the compounds.

- Protective clothing should be worn which includes closed toed shoes, long pants, and long shirt (or lab coat) when working with chemicals. When first opening a bottle of hormone in dried powder it is recommended to wear a mask and protective eyewear, as well. Dried powder under compression can aerosolize when first opened and accidentally be inhaled. This is not as much of a risk once the hormone is suspended in fluid.
- When first suspending the hormone in a solution (sterile saline, phosphate buffered media or sterile water, etc.), use a syringe to add the diluent through the stoppered opening. **Do not remove the rubber stopper** to add the diluent. When in powdered form, the chemical can be released in the form of fine solid particulates and inhaled upon opening. If the bottle is too small to hold the amount of solution needed for an injection, a lesser volume (keep track of the amount) can be injected into the bottle to re-suspend the powder and the solution removed from the bottle. Once in solution, it is possible to bring the hormone up to an appropriate concentration.
- In case of a spill, always place paper towels or some other absorbent material under the area where the injections are being performed so that chemicals are not left on the workspace. Make sure to wipe down the work area when finished with 70% ethanol to decontaminate the workspace, just in case the solution has splashed.
- Anyone who is pregnant should not be working with hormones or taking care of hormone-treated animals. The possibility of an interrupted pregnancy from exposure is not worth the risk to mother and child, regardless of safety precautions.

**4.2 Storage**

Hormones should be stored as outlined by the manufacturer to maintain product integrity and shelf-life. The two hormones most commonly employed, LHRH and hCG, will come as lyophilized powder while others may come prepared in a buffer ready for dilution or administration at a prescribed dosage. The manner in which hormones are received may depend on whether the hormones are protein hormones or steroids; the specific manufacturer; the amount, concentration, and/or intended use; and many other considerations. Lyophilization of protein hormones generally improves stability through removal of water and it also decreases mobility of the compound that might enhance chemical and/or physical degradation.

Protein hormones are also prone to degradation from proteases, and too vigorous shaking or vortexing when dissolved or diluted can create physical shear forces that will destroy some of the biological activity. Moreover, if not stored appropriately, proteins are susceptible to proteolysis and may be affected by interactions with other compounds in storage media or buffer that may lead to a further loss in biological activity. Protein hormones can usually be stored in solution for up to 1 month at 4 C in a refrigerator. For long-term storage (more than one year), it is generally recommended that proteins be maintained at -20 C to -80 C or remain as a powder until use (usually also stored in a cooled or frozen environment).

Steroid hormones (e.g., progesterone) are generally more stable and resilient than protein hormones (e.g., hCG and LHRH); however, storage in containers that prevent evaporation of alcohol-based (ethanol or methanol) or similar diluents help maintain steroids concentrations, and these should also be stored in cold or frozen environments long-term. Below are the recommended storage environments for hCG and LHRH.

- LHRH: Do not make up the LHRH until ready to use. Dilute the entire contents of the bottle even though you will only use a small portion of it (the µg quantities you need are too small to weigh out). Allocate into small individual eppendorf tubes and store

these tubes at -80 C (ultralow freezer) if possible; veterinary hospitals or labs often have an ultralow freezer. If an ultralow freezer is not available, aliquots can also be stored in a normal -20 C freezer. Store within a sealable bag with the following information written with a permanent waterproof marker: date, hormone, initials of preparer, the concentration/volume (e.g., 300 mg/200 µl), and volume in aliquot. If the hormone is stored in an ultralow freezer, the hormone is good for two to three years; if held at -20 C, the hormone should not be used after one year. Only remove from the freezer the number of aliquots needed for injections. The protein LHRH is a decapeptide and is the smallest functional protein known in vertebrates; hence, it is easily degraded and loses functionality over time.

- hCG: As discussed previously, do not make up hCG until ready to use, diluting the entire contents of the bottle to the appropriate dosage. This dosage will require calculation of the specific volume ahead of time depending on what is needed (see Section 5 for dosage calculations). It is not recommended to store hCG long-term in the freezer once in suspension, rather store it in the refrigerator at 4 C for one to two months. Using permanent waterproof marker, write the following information: the date that the hormone was prepared, initials of the preparer, the concentration/volume (e.g., 300 IU/200 µl), and the total volume of the aliquot. Dispose of unused hormone after the two-month expiration, as detailed in Section 4.3.

### 4.3 Disposal

As accidental exposure to reproductive hormones through skin contact, inhalation, mucous membranes, skin puncture, or other means may have reproductive consequences to those exposed, taking precautions during the disposal process is equally important. Therefore, all hormones and their storage containers should be disposed of as biological hazardous waste. Disposal of unused hormone and empty bottles (potentially carrying residual powder or liquids) should be in accordance with manufacturer recommendations and in compliance with local, state, and federal laws. This is usually detailed under *Disposal Considerations* on MSDS sheets. The importance of disposal of unused hormones is underscored by recent studies which have documented the impact of household pharmaceutical contents in municipal solid waste and sewage treatment systems which have the potential to impact biological systems in the environment (Musson and Townsend, 2009). As conservation-minded institutions rearing aquatic species which are sensitive to environmental contaminants, following hormone disposal criteria sets a responsible tone for the facility and does not contribute to this mounting problem of incidental pharmaceutical compound release.

- All syringes and needles should be disposed of in a certified, hard-plastic Sharps Box. These can typically be found in the institutions' veterinary facilities. Seal Sharps Boxes securely upon disposal and treat as biomedical waste.
- Glass vials used to store the hormones should also be disposed of within a Sharps Box. Gloves and paper towels should be placed into standard waste containers with plastic liners designated for the landfill. It is encouraged to discuss methods/protocols for disposal of hazardous chemicals or waste with veterinary staff at your facility.
- Do not pour unused hormone down the sink to enter into the local water sources. Once empty, place glass bottle in a larger plastic container, seal, and dispose of in the city landfill.

## 5. CALCULATING HORMONE DOSAGES

Lyophilized (dried powder) hormones arrive from the manufacturer in glass vials labeled with the total amount of hormone contained within the vial in International Units of Activity (IU) or in units of mass (g = grams). A concentrated stock solution is usually made from this powdered form by adding a specified volume of buffer solution (sterile saline, phosphate

buffered media, sterile water, etc.). Once the powder is dissolved or suspended into solution form, it can be aliquoted for storage into smaller amounts that correspond to the amount of hormone used for an experiment. This allows for a very cost effective and user-friendly way to manage hormone stocks by removing only what is needed.

This section will demonstrate the process beginning with creating stock solutions and aliquots from lyophilized powders, through to generating appropriate administrative dosages for hormone therapy. Before making up a hormone solution, the reader should consider the following questions:

- What medium will I use to suspend the hormone?
- What volume of fluid do I want to inject?
- What will be the final working concentration of hormone (e.g., 15 µg LHRH per animal)?
- How many dosages are in a bottle once I have determined the concentration of hormone (i.e., how many animals can I treat)?
- Will I inject a standard concentration across the board or modify according to body weight (BW)?
- How do I calculate how much buffer to add to the bottle of hormone?

### 5.1 Which Medium to Use

There are many different mediums that can be used to dilute dried exogenous hormones. The most common mediums are sterile: 1) 0.9% saline; 2) Simplified Amphibian Ringer (SAR) solution; and 3) phosphate buffered saline (PBS). Sterile saline is a common diluent found in all veterinary hospitals and should be easy to obtain for most zoological institutions. The formula for making SAR is shown to the right and consists of sodium chloride, potassium chloride, calcium chloride, and sodium bicarbonate that can be made in the lab or purchased[6]. PBS can be purchased[7]; the tablets should be dissolved in 100 ml sterile deionized water and stored in the refrigerator. Another option for medium is sterile deionized water; however, since water lacks the buffer capacities of PBS and SAR, it is recommended to use these first if they are available. If purchasing mediums from a chemical company, they will arrive pre-sterilized; however, if the mediums are being made in-house, it is important to sterilize them prior to suspension of hormones. It is recommended to discuss appropriate protocol and equipment for sterilizing media with veterinary or research staff in advance of any procedure.

**Simplified Amphibian Ringer Solution**

| Mix the following in 1 liter of distilled water | |
|---|---|
| Sodium chloride (NaCl) | 6.60 g |
| Potassium chloride (KCl) | 0.15 g |
| Calcium chloride (CaCl2) | 0.15 g |
| Sodium bicarbonate (NaHCO3) | 0.20 g |

### 5.2 Injection Volume

Once the suspension medium has been selected, it is important to understand what volume of fluid should be injected into the subject animal. This decision will impact all of the subsequent calculations and determine the amount of medium that will be added to the bottle of dried hormone. The volume to be injected depends upon the body size of the amphibian. Although it is preferred to minimize the injection volume, larger volumes result in increased accuracy of the measurement; i.e., there is a fine line between injecting enough solution so that the syringe hash marks can be read and the volume is accurate, while not injecting too much fluid into the animal such that medical complications arise. At the Memphis Zoo, the subject toads and frogs are primarily in the 25-60 g range and it has been found that injecting

6 Fisher Scientific Co. (catalogue #50-980-243)
7 Sigma-Aldrich® in tablet form (catalogue # P4417)

approximately 200 µl of hormone intraperitoneally has resulted in few complications. If working with anurans smaller than 25 g, the volume should be reduced accordingly.

Injections of small volumes of hormone at the required concentrations may not be feasible where very small animals are concerned (e.g., <3.0 g). Therefore, making up hormones at the correct concentrations and then applying topically to the skin or submersing the animal in the hormone solution may be another approach. Very few studies have looked at topical application of hormones (Rowson et al., 2001; Ogawa et al., 2011) and none of these studies used small anurans. Recently, a study by Ogawa et al. (2011) showed that immersing *Xenopus laevis* in water that had been treated with hormones induced ovulation in females with similar fertilization rates to those that had been given hormone injections. This finding suggests that there may be alternative methods for administering hormones to small anurans where injections are not feasible.

As a reference, the following table shows how much sterile saline is added to a bottle of hCG depending on the injection concentration desired (e.g., 100, 300, or 500 IU) in order to have the correct amount in 200 µl volume (Table 1). hCG can be purchased in different amounts of IU and this will impact how much medium is added to obtain the final injection concentration per volume.

| **Table 1.** | **Amount of IU hCG found in commercially distributed bottles and milliliters needed for resuspension** | | |
|---|---|---|---|
| Amount of hCG hormone and volume for priming or ovulation in anurans | milliliters sterile saline 2,500 IU hCG bottle | milliliters sterile saline 5,000 IU hCG bottle | milliliters sterile saline 10,000 IU hCG bottle |
| 500 IU / 200 µl* | 1 | 2 | 4 |
| 300 IU / 200 µl** | 1.665 | 3.33 | 6.66 |
| 100 IU / 200 µl*** | 5 | 10 | 20 |

Depending on the volume the bottle holds (e.g. 10 ml) you may need to dilute the hormone with saline and then transfer to another bottle to bring up to the correct volume. For example, if you are making up a concentration of 100 IU / 200 µl and you purchased bottles that are 10,000 IU, you will not be able to add all 20 ml of saline needed due to the bottle only holding 10 ml. In this case resuspend the dried powder in 8 ml of saline, remove to another empty bottle and then add the remaining 12 ml of saline to reach the 20 ml volume.
*Ovulatory dose for females; **spermiation dose for males; ***priming dose for females.

IMPORTANT: It is critical to use only one syringe and needle per animal when administering injections. Do not reuse the same syringe and needle for multiple animals. This can cause the spread of disease if one animal is infected with a communicable virus or fungus. Once the hormone dosage has been calculated, draw up all of the needed syringes at one time for all of the animals being treated, rather than going back and forth between animals and filling up the syringes.

Table 2 illustrates how much sterile saline is added to a bottle of Sigma-Aldrich® LHRH (#L4513) for a standardized injection and is not based on body weight (for calculations based on body weight, see section 5.4). Similar to hCG, LHRH can be purchased in different concentrations (1, 5, and 25 mg). A stock concentration of LHRH can be made, and then the amount can be varied depending on how the LHRH is being used (*priming females, **male spermiation,***female ovulation). For example, the authors would add 2.5 ml of saline to a 1 mg bottle and freeze 50 µl aliquots in eppendorf tubes for long-term storage (i.e., there would be 50 small vials of LHRH placed into the freezer). Thus, if treating males with 4 µg/10 µl of LHRH, one vial would be thawed and essentially 4 animals (total used = 40 µl) could be treated, leaving a little bit extra for fluid loss in the syringes. If treating a male with 16 µg/40

µl only one injection could be drawn from an aliquot. Do not re-freeze these small vials but discard any extra fluid.

**# of LHRH treatments per vial = µg of hormone in vial / µg of hormone per treatment**

| Table 2. | Amount of µg LHRH found in commercially distributed bottles and milliliters needed for resuspension | | |
|---|---|---|---|
| Amount of LHRH hormone and volume for spermiation, priming or ovulation in anurans | milliliters sterile saline 1 mg LHRH bottle | milliliters sterile saline 5 mg LHRH bottle | milliliters sterile saline 25 mg LHRH bottle |
| 1 µg / 2.5 µl* | 2.5 | 12.5 | 62.5 |
| 4 µg / 10 µl** | 2.5 | 12.5 | 62.5 |
| 8 µg / 20 µl** | 2.5 | 12.5 | 62.5 |
| 16 µg / 40 µl*** | 2.5 | 12.5 | 62.5 |

The following table is what the authors use in their lab to make up their LHRH stock concentration. For LHRH, a stock concentration is made using the volumes described above and 50 µl is aliquoted into small eppendorf tubes for long term frozen storage. In order to inject the correct concentration of LHRH into an animal, the volume of hormone injected is changed (this can also be calculated on a body weight basis). Depending on the volume the bottle holds (e.g. 1-5 ml) you may need to dilute the hormone with saline and then transfer to another bottle to bring up to the correct volume. For example, if you are making up a concentration of 4 µg/10 µl and you purchased bottles that are 5 mg you will not be able to add all 12.5 ml of saline needed due to the bottle holding less. In this case, resuspend the dried powder in 1 ml of saline, remove to another empty bottle and then add the remaining 11.5 ml of saline to reach the 12.5 ml volume.
*priming dose for females; **spermiation dose for males; ***ovulatory dose for females.

### 5.3 Number of Animals That Can Be Treated

There is typically a small amount of fluid remaining in the needle and end of the syringe after an injection. With each administration, this small amount of fluid eventually totals the equivalent of one full treatment lost per bottle, as compared to the initial calculation. Thus, when estimating how many animals can be treated from the hormone suspension, always reduce the number by at least one, as is evident in Table 3. This table shows how many animals can be treated with hormone, based on the hCG suspension volumes used in Table 1.

| Table 3. | Number of Animals Treated | | |
|---|---|---|---|
| Amount of hCG hormone and volume for priming or ovulation in anurans | Animals treated per 2500 IU hCG bottle | Animals treated per 5000 IU hCG bottle | Animals treated per 10,000 IU hCG bottle |
| 500 IU / 200 µl | 4 | 9 | 19 |
| 300 IU / 200 µl | 8 | 16 | 33 |
| 100 IU / 200 µl | 24 | 49 | 99 |

When calculating how many animals can be treated from a resuspended bottle of hormone (in this case hCG), it is important to understand there is always some small loss of fluid in the needle when the injection is completed and this loss of volume is repeated with each change in syringe. Therefore, it is not feasible to get out of the bottle the exact amount of injections originally calculated. For example, in the table above, instead of five treatments of 500 IU hCG from a bottle of 2500 IU, the authors subtract one treatment and recalculate that hormone can be administered to four animals.

Occasionally, hormone cocktails are used for ovulation, as described previously. In these instances an injection of hCG will be given first by itself (e.g., 500 IU/200 µl) followed by an injection of LHRH (e.g., 16 µg/40 µl), or they may be combined in one tube so as to administer one injection of 240 µl (200 µl hCG + 40 µl LHRH) instead of two separate injections. When combining the hormones into one tube, make sure to make up extra hormone so that you have enough for all the injections with some to spare.

It is important to use the smallest gauge needle possible when administering hormones. A 29-gauge needle that can be found at local drug stores and are commonly sold for diabetic treatment is recommended. A smaller 0.3 cc or 0.5 cc syringe will allow for more accurate measurements. Make sure to remove all air bubbles from the syringe by gentle tapping and use the plunger to draw out the required volume (Figure 6).

**Figure 6:** Proper techniques for using a syringe.

### 5.4 Units of Measure

In order to administer hormones safely to animal subjects, it is critical to understand the units of measure for calculating dosages. If not familiar with the units of measure at which hormones are commercially distributed, review calculations with a resident veterinarian or research staff member, as it is very important to calculate the hormone dosages correctly. Common conversions necessary for calculating hormone concentrations are as indicated in the blue box to the right. The final working concentration of hormone will vary based on species, gender, size, and response levels.

**Pay Attention to the Numbers and Orders of Magnitude**

1 gram = 1000 milligrams (mg)
1 milligram = 1000 micrograms (µg)

1 liter = 1000 milliliters (ml)
1 ml = 1000 microliters (µl) = 1 cc

Hormones are very potent - a little can go a long way. Too much can be very dangerous or even deadly to the animals.

<u>Hormone Dose Independent of Individual Body Weight (Standardized Method)</u>

There are two basic methods to calculate and determine the amount of hormone to administer to each individual animal. The first technique commonly used is a *standardized method* that determines the concentration based on the average body weight (BW) of the entire treatment group (see calculation below). Subsequently, the same concentration (and volume) is given to all of the animals based on the group's average body weight. This is common in labs where extensive experience with particular species has been well documented. However, when beginning a study to characterize a hormone's efficacy on a new species, this technique is not recommended. An advantage of this method is that the same stock solution is used for all animals, decreasing preparation time and variation that might result from preparation of separate injections. A disadvantage to this method is that the hormone dose is calculated based on the average body weight of the entire group, thus the amount of hormone administered to each individual is constant, but the concentration per gram body weight may differ from when the hormone dose is calculated from each individual's weight.

$$\frac{\text{Individual 1 BW + Individual 2 BW + Individual 3 BW, etc.}}{\text{Total number of individuals}} = \text{average body weight}$$

**Standardized Method Example**

If the hormone dose is 0.1 µg LHRH / g body weight, then 0.1 µg of hormone is used for every gram of the group's average body weight. If the average body weight = 40 g, then 4 µg of hormone will be used (0.1 µg hormone x 40 g body weight = 4 µg hormone) for each animal regardless of their individual weight.

Using Table 2, each animal would be injected with 10 µl of LHRH.

Hormone Dose by Individual Body Weight (Body Weight Method)

To determine a hormone dose based on the individual animal's body weight, it is necessary to calculate an average weight for the entire group (e.g., 0.1 µg LHRH/g body weight), similar to the standardized method described above. Subsequently, the volume of hormone drawn up into a syringe is varied to obtain the correct hormone concentration depending on the animal's weight. The advantage of using this method is that the hormone dose is specific to each individual and normalized, such that all of the animals receive the same concentration of hormone, minimizing variance within treatment groups. The disadvantage of using this method is that it takes significantly more time to prepare customized hormone doses for each animal, due to necessary animal weighing and hormone concentration recalculation every time. In other words, the amount of hormone must be adjusted based on the animal's weight and different volumes taken from the stock in order to obtain the correct concentration; a single stock concentration is made, as described in Table 2, and to obtain the desired hormone concentration requires drawing up different volumes in the syringe.

Perhaps the greatest disadvantage to using the *body weight method* is that when working with hormones in small volumes (e.g., 4 µg LHRH/10 µl), small changes in weight are difficult to measure accurately in a syringe. Table 4 illustrates this problem through an example where the difference in volume that must be administered to a 40 gram toad compared to a 41 gram toad is 0.25 µl (10 µl vs. 10.25 µl). The total difference between a 40 gram toad and a 50 gram toad is only 2.5 µl. When working with larger volumes of hormone, such as the hCG in 200 µl volumes, it is easier to make these adjustments and measure the volume accurately. In this instance, very large volumes may have to be administered to an animal or separate stocks would have to be made.

| **Table 4** | **Volume of LHRH (µl) that must be administed to Fowler toads weighing between 40-50 grams in order to give each animal 0.1 µg / g body weight.** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Grams Body Weight of Fowler Toad | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 |
| Volume LHRH (µl) administered to reach 0.1 µg / g body weight | 10 | 10.3 | 10.5 | 10.8 | 11 | 11.3 | 11.5 | 11.8 | 12 | 12.3 | 12.5 |

Hormone Dose by Individual Body Weight without Changing the Volume Administered

Perhaps the most widely used method for calculating hormone dosage is based upon individual body weight while keeping the volume of fluid administered the same between animals. Prior to beginning an experiment, prepare stock aliquots of the hormone (e.g., 2.5 µg/µl stock LHRH) that can be stored in 50 µl aliquots at -80 C for later use. Use the steps that follow for each animal (the example below uses an LHRH concentration of 1 µg/µl as the amount to be administered per body weight; however, that value is only given as an example and by no means are the authors dictating that this is the concentration one should use for every situation):

| STEP | EXAMPLE |
|---|---|
| Calculate the amount of hormone needed per individual. | If administering 1 µg LHRH /g to a 30 g toad, one would need 30 µg LHRH |
| To determine the individual's dosage, divide the amount of hormone needed per individual by the amount of hormone solution to be injected. | If 200 µl hormone solution is needed, then:<br>30 µg ÷ 200 µl= 0.15 µg/µl |
| Further divide this number by the stock aliquot concentration available. | 0.15 µg LHRH/µL ÷ 2.5 µg/µL stock LHRH = 0.06 |
| To determine the desired stock volume, multiply the previous calculation by the amount of hormone needed per individual plus extra for pipette error. | 200 µl injection volume + extra volume for pipette error, or approx. 300 µl; then: 0.06 x 300 µl = 18 µl. Accordingly, one would need to prepare 18 µl of stock LHRH + 282 µl of medium (i.e., SAR, sterile saline, or sterile water) to total 300 µl |
| Place all of this into a small eppendorf tube and draw out the amount needed for the individual to be injected, administering the exact hormone dose to the research animal. | Draw out 200 µl from the eppendorf tube containing the 300 µl and administer to the animal. |

## 6. HORMONE ADMINISTRATION

Administering hormones to amphibians should only be conducted by those trained in the safe use of syringes and needles, and the restraint and injection of animals. It is recommended that the institution's veterinarian, researcher, or curator inject the amphibian or provide one-on-one training for any other staff members, such as keepers. It is imperative that adequate training be provided as there is a risk to the animal's health if the needle is too large, placed incorrectly, or pushed in too deep. Keepers trained in administering injections are also very capable of performing this, once approved by the veterinary staff. The use of a 0.5 cc or 1.0 cc syringe is suggested as the syringe hash marks are easier to read and measuring the volume is more accurate. It may be tempting to reuse syringes and just change needles, but best practices suggest that each individual syringe is disposed of after a single application.

Intraperitoneal (IP) – injecting the hormone into the body cavity from the side of the animal.

Intramuscular (IM) – injecting into the muscle layer, usually the leg of the frog.

Subcutaneous (SQ) – injecting under the skin, but not in the muscle.

Dorsal lymph sac (DLS) – injecting into the space on the back of the frog, lateral to the spine and towards the rump.

The size (bore) of the needle used is critical, and using the thinnest needle possible is preferred. Diabetic needles can be purchased at local drugstores in the U.S., with similar options available in other countries. The use of 28-30 gauge needles is recommended. The gauge of a needle refers to its width; the higher the gauge of a needle the smaller the width. The use of a larger needle (smaller gauge) may increase the risk of infection at the injection site.

There are several routes of administering an injection, each of them with different locations and methods of effectiveness. The most common injection sites are intraperitoneal (IP), intramuscular (IM), subcutaneous (SQ), and into the dorsal lymph sac (DLS). Figure 7 shows a few of the more common injection sites on a frog *[Lithobates* (=*Rana) pipiens]* or toad *[Anaxyrus* (=*Bufo) fowleri]*. It is recommended that hormone injections be given IP or through the DLS, as these routes of administration have been shown to be the most effective. A direct comparison between these two routes of administration on sperm parameters or ovulation effectiveness has not been conducted to the authors' knowledge. Though, a study by Obringer et al. (2000) showed that *[Anaxyrus* (=*Bufo) americanus]* receiving an LHRH IP

injection produced more sperm than via an SQ injection. A follow-up study by Rowson et al. (2001), tested whether *[Anaxyrus* (=*Bufo) americanus]* could absorb LHRH through dorsal or ventral application (without injection) which resulted in no animals producing sperm when applied on the dorsal surface and some animals producing sperm when LHRH was applied to the ventral surface, although sperm concentration was less than if the hormone had been administered intraperitoneally. More studies are desperately needed to develop protocols for dermal (skin) absorption of LHRH. Many frogs are so small (3-5 g) that an injection is not feasible even with the smallest gauge needles because the perforation created into the animal would be too large.

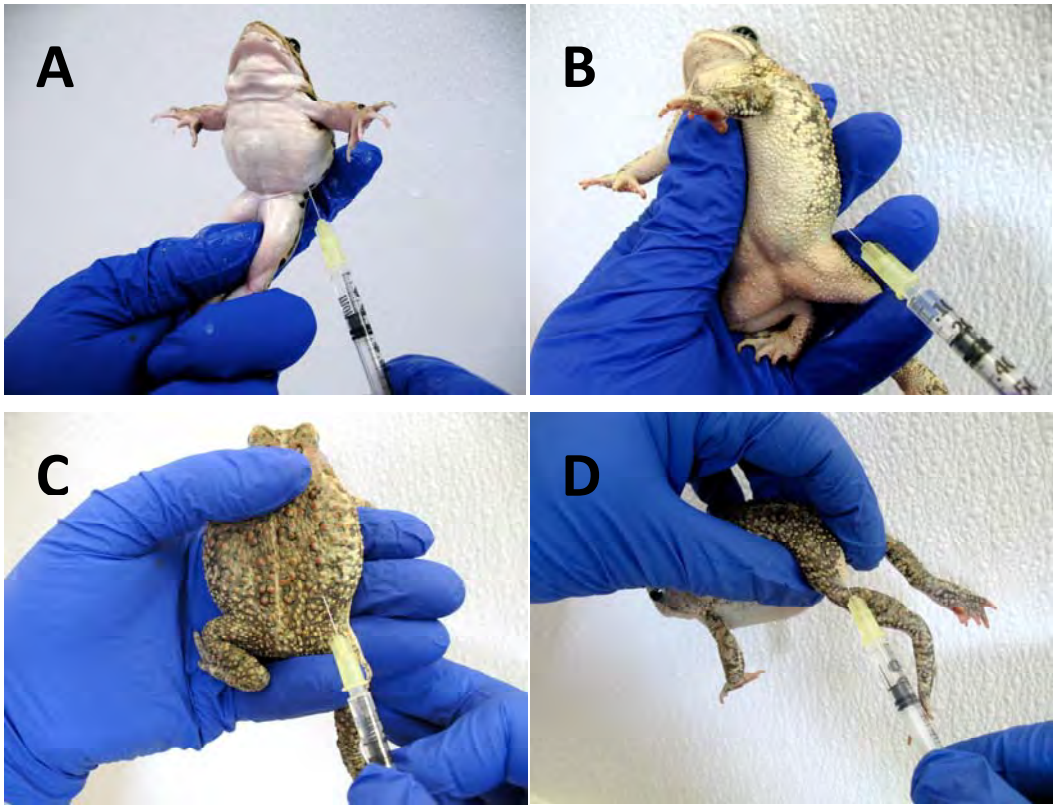


**Figure 7.** Restraint and hormone administration in leopard frogs *[Lithobates* (=*Rana) pipiens]* and Fowler toads *[Anaxyrus* (=*Bufo) fowleri]*. **A:** *Frog on a stick* method of administering an intraperitoneal (IP) injection to leopard frogs. The legs must be stretched out and restrained tightly to avoid escape. The injection area is low and on the ventral side of the animal; the pointer finger is placed on the animal's back to counter the injection pressure. **B:** An IP injection on a Fowler toad. The injection is low and on the ventral side, similar to leopard frogs. The pinky finger is used to restrain the hind leg so that the toad does not interfere with the injection. **C:** Dorsal lymph sac (DLS) injection on a Fowler toad, with the pinky finger restraining the animal so it is unable to escape. **D:** An intramuscular (IM) cradle injection on a Fowler toad.

When administering an injection, the needle should only be inserted enough to reach the desired location and it is not necessary to push the needle all the way to the plastic. As an example, for an IP injection the goal should be to deposit the hormone in the body cavity space, not deep into an organ. If conducted correctly, the chance of injuring the animal is minimal. The risk of animal injury or death would more likely result from miscalculating the correct amount of hormone, rather than complications at the injection site. By giving an IP injection in the side of the animal near the hip, most of the vital organs are avoided and injection is more likely to enter directly into the ovary or testis, which are the intended targets, if using hCG.

Once the injection site has been reached, slowly dispense the hormone and hold the injection for two seconds to insure that the fluid has dispersed. If the needle is removed too quickly after injecting, the fluid may come out with the needle before it has dispensed, which will affect the animal's response. It is important to remove the syringe in the same direction you injected; if the needle is tilted or pulled out at an angle, the skin and muscle will tear, risking loss of the hormone out of the opening and damage to the animal. Upon withdrawal of the needle, it is critical to rub the injection site immediately before setting the animal down to close off the hole and prevent seepage of hormone. For this reason, it is important to wear gloves, as rubbing the skin at the injection site could lead to direct contact with any hormone that may have leaked. Ranids are extremely difficult to handle due to their smooth, wet skin; a damp paper towel can help with restraining frogs.

***The authors would like to end this section with a brief note of caution:*** There is not a one-size-fits-all hormone protocol for amphibian species, and special care should be taken when adapting current protocols to new species. This may be particularly true for smaller amphibians where overdosing through miscalculation might be easier given the extremely small volumes of hormone being used. Some of these hormones (e.g., neurotransmitters) play important and sensitive roles in multiple physiological processes, and the potential repercussions of these substances on the animal as a whole are still unknown; administration of LHRH, hCG, or dopamine antagonists have been linked to the deaths of a few animals from several species (pers. comm. A. Kouba). Different species of amphibians will have widely diverse metabolisms, so the hormones used will not be equally effective even when dosages are calculated based on body weight.

**Guidelines for Testing Hormones on New Species**

1. Start with a low hormone dose calculated on a body weight basis, then scale back.
2. Time injections apart by more than a week.
3. Test on one animal or pair of animals before applying to others, especially if the animal is endangered.
4. Test LHRHa before moving on to hCG.
5. Keep in mind that the response time to the hormones will be widely variable, impacting natural breeding or gamete collection for IVF.

## 7. FREQUENCY OF HORMONE USE

A common concern when using hormones on animals is how often (frequency) they can be administered safely and still obtain a physiological response (i.e., sperm produced or eggs ovulated); too high of frequency has induced an immune response in mammals, making additional treatments unsuccessful. LHRH is too small of a protein to elicit an immune response; however hCG is a large polypeptide that could possibly generate an immune response resulting in the production of antibodies. Over time, the overuse of hCG in mammals becomes ineffectual due to the animal's immune response (Swanson et al., 1995). To the authors' knowledge there are no detailed studies showing this to be the case for amphibians. In the authors' experience, Fowler toads have received hCG injections every couple weeks for years at a time with no observable effects on sperm production (unpublished), suggesting that the same immunogenic attack on the hCG molecules does not occur in amphibians as it does in mammals. Furthermore, anecdotal evidence from several captive breeding programs (e.g., Wyoming toad and Puerto Rican crested toad) indicate that repeated injections on males and females over multiple breeding seasons continue to be equally effective over time. However, researchers do not yet know the impact of hormone overuse on the lifespan of

species commonly used in research, much less the short-term health effects that the hormones might have on species that have not been studied.

From reviewing seasonal breeding logs for some amphibian programs, it has become evident that when animals receive injections too frequently within a breeding season, there may be a negative impact on sperm production in males and ovulation in females. When sperm concentration of Fowler's toads was tested experimentally by the authors, results indicated the following (Figure 8):

1. Sperm concentration did not seem to be adversely affected by less frequent hormone administration.
2. A few injections close together are effective without immediate negative consequences; however, sperm concentration begins to decrease by the second week, showing that this hormone regime was not sustainable.

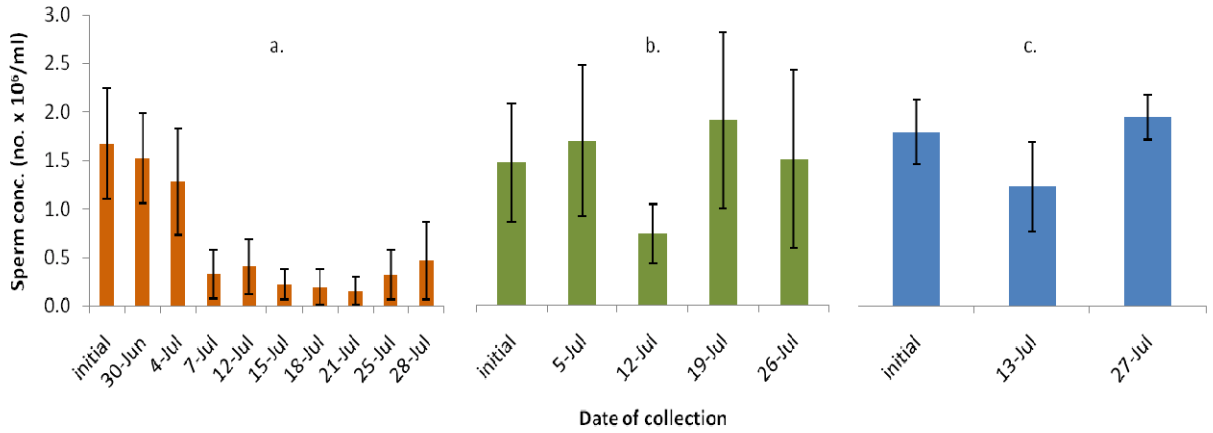


**Figure 8.** Concentration of sperm from Fowler toads administered 300 IU hCG at different frequencies. Treatment groups (n = 5 each) were: **a**. toads injected with hCG twice weekly; **b**. toads injected once weekly; **c**. toads injected every two weeks. Data are mean 3 SD; preliminary (unpublished) and not statistically analyzed. Not shown is the data from toads injected every three weeks, as these results are similar to the data shown above in blue (every two weeks).

The decrease in hormone effectiveness when administered too frequently in males is likely a result of one or more of the following: 1) receptor desensitization or degradation; 2) receptor down-regulation; or 3) sperm depletion. Problems with multiple hormone injections arise in captive programs when there are fewer males than females, resulting in males being injected with hormones on multiple occasions to fertilize the females' eggs as they are spawned. This can also be a problem in laboratories studying these reproductive processes, as it is tempting to organize more frequent experiments rather than waiting.

When hormones are injected too frequently, receptors for that particular protein become desensitized or down regulated, which means the animal becomes less responsive to the treatment. It is recommended to wait at least one week between hormone injections.

Females occasionally need priming (Figure 9) and multiple injections to complete egg maturation and stimulate ovulation (due to missing environmental cues), yet the appropriate spacing of injections that will not compromise the hormone's effectiveness is not always known. Therefore, this should remain an active area of investigation.

## 8. PRIMING HORMONES AND HIBERNATION

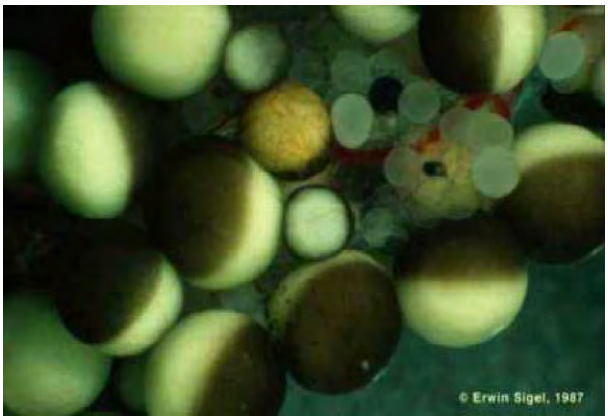


**Figure 9**. The amphibian ovary has eggs at various stages of developmental competence as shown here. The goal of priming a female is to complete the ripening of those eggs that are too small and have either not gone through vitellogenesis (eggs with no yolk) or not completed final growth and maturation.

Because amphibians are ectothermic (poikilotherms), temperature is the most important environmental factor controlling reproduction and the release of hormones, especially in temperate species. There is some evidence that photoperiod may also play a role in a few anuran species, but only if temperature is appropriate for reproduction (Rastogi et al., 2011). Rainfall levels contribute to reproduction in desert, semi-desert, and tropical geographic anurans, yet it is unclear if the drop in temperature or barometric pressure associated with the rainfall is the driving force for reproduction or if the rain itself is the underlying stimulator. Unfortunately, hormonal data for wild anurans in relation to temperature is limited and much of what is predicted is based on generalizations for species whose reproductive ecology is only partially understood. Most anurans reproduce with a predictable cycle of gametogenesis and spawning, typically breeding once a year. The various environmental stimuli that occur during the year long reproductive cycle act to stimulate the various stages of endogenous hormone release followed by gametogenesis. There is very little information known regarding salamander and caecilian reproductive cycles and their relationship to environmental parameters. Much of what will be discussed in this section is based on what is known about frogs and toads, underscoring the need for more research on salamanders using hormone stimulation.

In the wild, some male and female amphibians will have periods of interrupted spermatogenesis or oogenesis during the winter, or in the summer following breeding. However, amphibians maintained at a constant ambient temperature can show continuous gamete development throughout the entire year. If the keeper/curator is mimicking the outside environmental changes in temperature for the holding area, there may be periods where the sperm or egg development is arrested. It is important to understand how the husbandry impacts sperm and egg output in response to hormones, as gametes may be difficult to obtain for research or breeding if an animal has been cooled down as a result of mimicking outside temperatures.

For many temperate and alpine species like the Wyoming toad, boreal toad, or mountain yellow-legged frog, a period of hibernation or cooling completes final maturation of the eggs in the female. If the eggs do not go through final maturation, a single hormone injection may not be successful in artificially completing this process, as the eggs are not ready to be ovulated. Hibernating or cooling amphibians is a general practice in captive husbandry and has been quite successful in stimulating animals to reproduce. Yet the exact humidity, length of hibernation, and minimum holding temperatures are not known for most species. Stress and a weakened immune system during the hibernation procedure have resulted in death primarily due to fungal and bacterial infections that overwhelm the animal. Certain species appear more susceptible to complications during hibernation and the loss of valuable animals has prompted many institutions to circumvent cooling and use hormones instead.

One of the most common reproductive dysfunctions in captive amphibians is the failure to finish gametogenesis in hibernation-obligate species. To complete egg maturation in females, it is necessary to use a series of hormone injections called primers, that when spaced out over

time, allow the eggs to finish development prior to ovulation. Although a series of priming hormones prior to an ovulatory dose of hormone can overcome this reproductive dysfunction and complete egg maturation, there is very little known about how often the primers should be given, what the appropriate time is in between priming injections, when maturation of eggs has completed, or which combination of hormones (e.g., hCG, LHRHa, or both) is the better primer for a given species. The authors have developed a priming regimen for American and Fowler toads that has proven quite successful in obtaining gametes from nearly 100% of the females (Figure 10) at any time of the year without prior hibernation. The protocol outlines how two low doses of hCG (100 IU) are given to a female prior to an ovulatory dose of hCG (500 IU) and LHRHa (20 µg). This cocktail combination of ovulatory hormones has worked better than either hormone alone. Eggs are spontaneously ovulated about 10-12 hours after the ovulatory injection (Figure 11). When collecting sperm for IVF, the male Fowler toads are given hCG (300 IU) only; however, if natural amplexus and breeding is attempted, LHRHa (10-15 µg) is combined with hCG in order to induce stronger reproductive behaviors (Kouba et al. 2009).

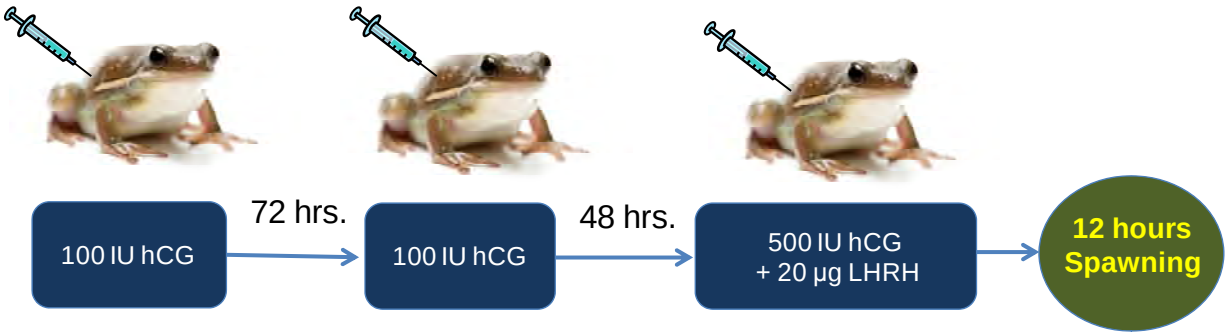


**Figure 10.** Memphis Zoo's priming protocol for inducing spawning in female toad species. First, toads are given a low priming dose of hCG (100 IU/200 µL) followed by a second priming injection of hCG (100 IU/200 µL) 72 hours later. The ovulatory injection is administered 48 hours after the second primer with a higher dose of hCG given in combination with LHRHa (500 IU hCG + 20 µg LHRHa, respectively, in 220 µL volume). Spontaneous spawning occurs 10-12 hours after the ovulatory injection, or sooner if the female is in amplexus with a male. Following the ovulatory injection, the female should be paired with the male if the goal is natural breeding and fertilization. Once the ovulatory injection is administered, females need to be placed into a water reservoir at a depth that covers at least half their body; water is needed for the production and hydration of egg jelly (necessary for fertilization). If the goal is to collect eggs for IVF, close watch of the female is required so the eggs can be collected immediately upon laying. If eggs are left in water too long (more than 15 minutes) the egg jelly hardens and becomes impermeable to sperm, reducing and/or eliminating fertilization potential.

**Figure 11.** Spontaneous ovulation in an American toad *[Anaxyrus (=Bufo) americanus]* following hormone administration (over 6000 eggs were deposited). Eggs can be moved into a Petri dish or multi-well containers for fertilization and evaluation.

If hormones are used to induce ovulation and spawning in a female and she fails to spawn her eggs due to a lack of sexual interest by the male (amplexing) it may become necessary to express her eggs so that the female does not become egg bound and die. Some species are more susceptible to this than others and eggs are clearly visible in the body cavity.

As detailed above, there will be variations to the priming protocol due to species-specific differences in the number of primer injections needed or the duration between injections to complete egg maturation. For example, it was found that out of nine Mississippi gopher frogs *[Lithobates* (=*Rana) sevosus]* treated with the priming regimen, none of the females laid eggs. However, when the same procedure was repeated four months later, all nine animals oviposited, leading to the production of over 1,400 IVF tadpoles (Kouba et al., 2012; Figure 12). It is possible that the first round of hormones did not have sufficient time to complete the egg maturation and that a resting period was necessary. The authors plan to test whether administering the two low priming doses without the ovulatory dose a few months in advance of the protocol outlined in Figure 10 will be equally effective for stimulating ovulation when next performing IVF for gopher frogs. Unfortunately, none of the female gopher frogs spawned spontaneously and all of the eggs had to be expressed through gentle squeezing from the body cavity. Females often fail to spawn even though they have ovulated eggs into the body cavity due to the absence of an amplexed male (who squeezes the female and initiates a cascade of hormones leading to spawning). One observation yet to be quantified is that captive females primed with hormones before the ovulatory dose lay more eggs than those that received an ovulatory injection only. This may be because the primer hormones recruited additional follicles for maturation and ovulation (i.e., egg numbers were closer to wild clutches).

**Figure 12.** Mississippi gopher frogs *[Lithobates* (=*Rana) sevosus]* were primed with hormones using the Memphis Zoo's protocol, resulting in hundreds of in vitro fertilized tadpoles and froglets produced. Eggs had to be expressed from the females and refrigerated sperm was used to fertilize them.

Of note is the observation that several low dose primer hormones may reduce the need for large concentrations of ovulatory hormones to induce spawning. Although there are more injections required, the potential for overdosing an animal is minimized by using primers; this is particularly important if the safety threshold is not known for a species. Primer hormones have been used successfully to breed *Lithobates (=Rana) pipiens* (Subcommittee on Amphibian Standards, 1996), *Xenopus laevis* (Subcommittee on Amphibian Standards, 1996; Green, 2010), *Anaxyrus (=Bufo) fowleri* (Kouba et al., 2009), *Lithobates (=Rana) sevosus*

(Kouba et al., 2012), *Anaxyrus (=Bufo) houstonensis* (pers. comm.. Paul Crump), and *Anaxyrus (=Bufo) baxteri* (Browne et al., 2006). When modifying any of the examples mentioned to develop a protocol for a new species, care should be given to adjust the amount of hormone depending on the mass of the animal. Another important consideration is the level of primer hormone administered.

The authors recommend using the primer hCG at 10-20% of the ovulatory dose that will be administered. The goal is to mature the full cohort of eggs from the resting pool in the ovary, but not to induce ovulation too early as there may be a small number of eggs that have already matured and are ready to ovulate. In the Memphis Zoo's protocol, hCG is used instead of LHRH, as even low doses of LHRH can occasionally cause some immature eggs to be laid before researchers are ready to stimulate natural breeding or conduct IVF. However, if LHRH is used for priming purposes, the same rule would apply: use 10-20% of the ovulatory dose as the primer concentration.

## 9. HORMONES FOR NATURAL BREEDING

The next two sections will provide information on how to use hormones for assisted breeding in anurans and, failing that, how to conduct IVF for species that will not amplex or which consistently produce unfertilized eggs. The optimal situation is to use hormones for 'kick-starting' the animals' natural reproductive behaviors and promote natural fertilization. Habituating these behaviors in species showing reproductive dysfunctions through the use of hormones may improve productivity of future mating attempts, possibly even removing the need for hormonal therapy. Ensuring a greater chance for reproductive success when promoting natural fertilization using hormones requires keeping in mind the aspects outlined in the following sections.

### 9.1 Egg and Sperm Synchronization

Synchronizing egg and sperm release using hormone therapy is perhaps the most challenging part of assisted breeding. It is not uncommon for institutions to report one or several circumstances: 1) the female laid eggs yet there was no male in amplexus, and thus no fertilization; 2) the male was seen in amplexus in the evening, yet the next day eggs were laid with poor fertilization and the male was no longer in amplexus; and 3) the female fails to lay eggs even though the male is in amplexus or she lays eggs 3-4 days later after the male has already unclasped. These three scenarios are all examples of situations where the release of eggs and sperm were not synchronized. Historically, male and female amphibians at zoological institutions have been injected simultaneously in the morning, but this injection protocol may benefit from some modifications aimed at better synchronizing the deposition of gametes.


**Figure 13.** Example of hormone injection schedule to elicit natural breeding and amplexus in American toads to occur overnight**.** Ovulation to occur 11-15 hours post-administration (PA) of hormone.

By studying the production of sperm and egg release over time for American, Fowler, and Wyoming toads in response to LHRH and hCG, researchers have a better understanding of the timing of injections in order to synchronize gamete release and optimize fertilization potential (Figure 13). When conducting natural breeding trials for American toads, a cocktail

injection of hCG (300 IU/200 µl) + LHRH (15-20 µg) is used for males (30-40 g toads) because it has been found that hCG induces higher sperm production, while LHRH encourages a stronger amplexus response. Although this injection regimen works well for American and Fowler toads, there may be a wide species-specific response to a cocktail formulation. Therefore, when trying to adapt a hormone regimen to a new species the hormone concentrations should be adjusted for bodyweight (BW) until more is understood regarding their physiological response. For female toads (bufonids) the priming regimen detailed above is applied using low doses of hCG only, followed several days later by administering an ovulatory injection of hCG (500 IU) + LHRH (15-20 µg). A higher concentration of hCG is used for the female than the male.

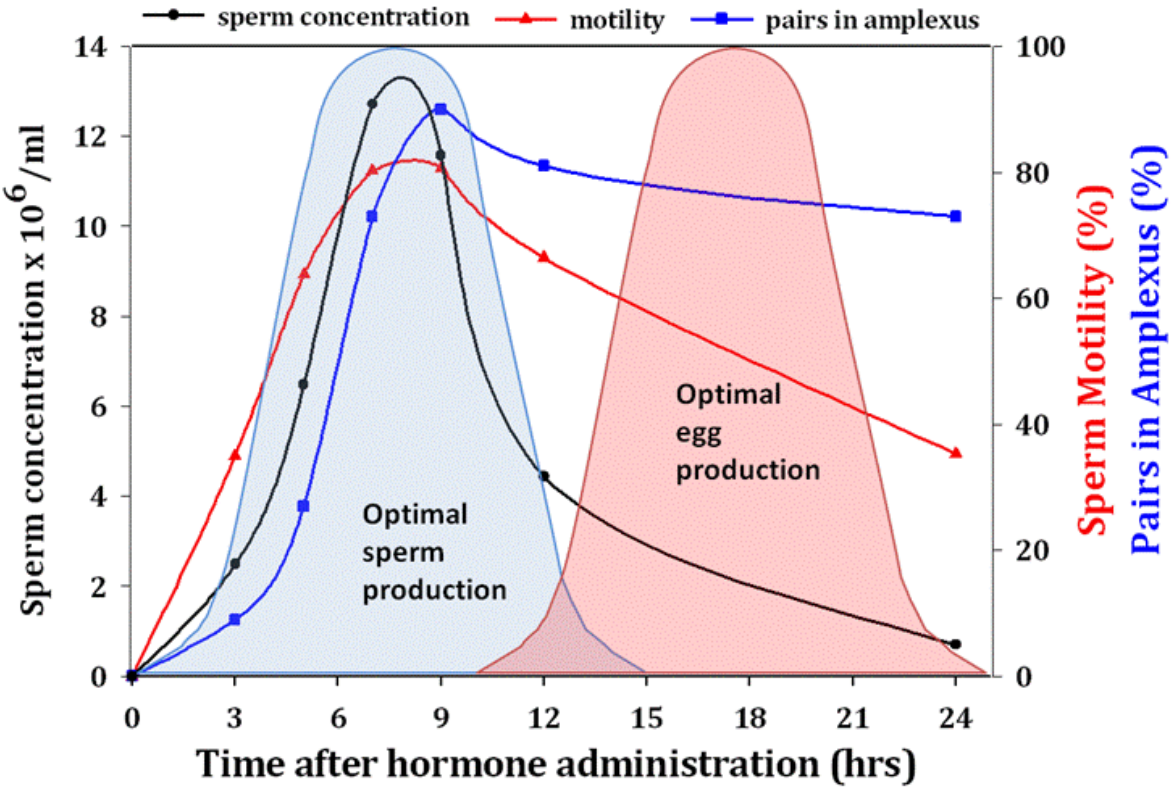


**Figure 14.** Sperm production, quality, and amplexus in male American toads *[Anaxyrus (=Bufo) americanus]* after stimulation with hCG (300 IU) + LHRHa (4 µg) in relation to egg spawning following hormone administration (500 IU hCG + 15 µg LHRHa). Note the asynchronous production of gametes when hormones are administered simultaneously. To synchronize sperm and egg release, a timed-breeding regimen is recommended where males are injected 8-10 hours after the females.

American, Wyoming, and Fowler toad males have a very prominent bell-shaped curve in sperm production with optimal concentrations occurring 5-7 hours post-administration (PA) of hormone and decreasing dramatically after 12 hours PA; however, female toads of the same species typically spawn 11-15 hours PA of the ovulatory dose (Figure 14). Thus, when males and females were treated simultaneously with hormones, sperm production was dramatically decreased by the time eggs were being laid. The number of fertilized eggs was greatly increased when hormone injections were staggered to take advantage of this timing phenomenon. Specifically, males are injected about 8-10 hours after the females are injected (i.e., females could be injected in the morning and males injected late in the evening if the goal is to have the female lay eggs overnight). By staggering the injections a timed-breeding protocol is created, which will likely work for other bufonids. More studies are needed to understand timed-breeding for other genera. For example, studies in the authors' lab for northern leopard frogs and Mississippi gopher frogs indicate that sperm production peaks

within 0.5-1 hour PA of hormone and decreases sharply 2 hours later (unpublished data); this earlier spermiation by male ranids is very different to the timing in bufonids. It is important to note that the Memphis Zoo studies are examining spermiation of non-amplexed males in response to hormones, and any variance in timing and duration of sperm release in amplexed males is unknown. Potentially, once the hormones induce the males to amplex, a natural endogenous hormone cascade is initiated that will extend the duration and quantity of sperm production.

**9.2 Importance of Hydration to Reproductive Success**

In nature, aquatic breeding anurans need a period of hydration in water to insure successful and optimal reproduction. Typically, mated pairs will amplex for several hours in water prior to spawning and fertilization. This period of hydration is necessary for males to absorb enough water to produce the spermic urine necessary to fertilize 4000-8000 eggs, while the females utilize the water to hydrate the egg-jelly necessary for fertilization. Spawned eggs produced without egg-jelly or that do not have good quality jelly will have poor fertilization rates. Exposure of sperm to egg jelly is required to induce the acrosome reaction (AR). When the AR is induced, biochemical changes to the sperm-head occur that allow them to bind to the egg surface. There is a cascade of events that follow the AR, most importantly the release of enzymes that allow sperm penetration of the egg.

Wild anurans reproduce quite successfully in response to environmental stimuli, but these cues are missing in captive environments and the animals may not always hydrate sufficiently for optimal production of sperm and/or egg jelly, even when amplexed. It is critical to make sure that aquatic breeding amphibians are immersed in water for the appropriate amount of time prior to spawning in order to hydrate. As most aquatic breeding tank set-ups also include dry land, even hormone-induced females may not hydrate sufficiently as a result of the lack of environmental cues, resulting in poor egg jelly or selecting to lay eggs on the land. Keepers also report low fertilization rates, possibly a reflection of males not being immersed in water long enough to produce sufficient spermic urine. It has been shown that hormone-induced male Fowler toads immersed in water over the course of 12 hours can produce enough spermic urine to equal 1/3 their total body mass.

The authors recommend that once animals go into amplexus they remain in water with no dry land available. This is not to say the mated pair is swimming, but that enough water is in the breeding tank such that the ventral side (drink pouch) of both the male and female are always submerged. Varying the depth of the water and providing plastic plants is also valuable for those species that may be stimulated to spawn eggs in specific water depths or require attachment sites to help pull the eggs out of the cloaca. If there is a concern, animals that fail to produce eggs after 24 hours can be moved to a dry location for a period and then placed back into the water. In summary, make sure the breeding pair is forced to hydrate in water and the depth is at least sufficient to cover the amplexed male's ventral abdomen.

**9.3 Assisted Amplexus**

Even after administration of hormones, some males will not amplex females and assisted amplexus may be necessary. Allow 3-4 hours to see if the males will amplex naturally before intervening. Sometimes males can be encouraged to amplex a female, by simply holding the male on the female's back until he clasps her. Another method is to hold the male toad over the female in an angled position such that his forelimbs are reaching out trying to grasp something, in this case the female. The authors have found the grasping behavior and angle of the hold produced by this positioning works much better than just holding the male on the female's back. Many problems with amphibian breeding could be rectified by these simple actions. As amplexus tends to occur in the evening, plans must be made ahead of time to readjust work schedules to accommodate these assisted breeding activities.

## 10. HORMONE USE FOR IN VITRO FERTILIZATION

There are some amphibians that despite every attempt to mimic environmental cues, play breeding calls, or administer various combinations and concentrations of hormones, still fail to breed naturally. For example, in spite of all measures to stimulate natural or assisted breeding for the critically endangered Mississippi gopher frog, males fail to amplex and females fail to spawn their eggs even though they have ovulated them into the body cavity. In such scenarios, the only option currently available is to use more invasive measures such as the collection of sperm and eggs for in vitro fertilization (IVF) (Figure 15).

**Figure 15.** Mississippi gopher frog produced by IVF.

The terms artificial fertilization (AF) and IVF are often used interchangeably by amphibian reproductive biologists, and in the literature, to denote the artificial insemination of eggs in a Petri dish. Although AF is probably a more appropriate term for anurans where external fertilization is more common, IVF is more appropriate for salamanders, newts, and caecilians that exhibit internal fertilization. For clarity purposes, IVF will be used throughout this section.

Conducting IVF is fairly straightforward in anurans compared to mammals, specifically because they display external fertilization in water. The most challenging aspect of the IVF procedure is the extraction of high quality eggs and sperm for insemination from live animals rather than euthanized ones. The use of IVF in amphibians has been detailed extensively for nearly 50 years. Thousands of articles have documented the employment of this technique on a few common species, although the animals were typically sacrificed for gamete collection. Knowledge pertaining to the use of IVF has been collected from several key laboratory species and is now being applied to the conservation of endangered species within the same genera (e.g., bufonids and ranids) though on live animals which have been hormonally stimulated to produce gametes. The collection of sperm from live males was first documented in 1976 (McKinnell et al.); however, more than 95% of the IVF studies recorded to date have utilized testes macerates from sacrificed males. Most likely, any attempts to conduct IVF using sperm and eggs from endangered live animals will be a first for that species.

Establishing IVF protocols for anurans depends upon having some basic knowledge and experience in: 1) hormone protocols useful for spermiation and spawning; 2) assisted and/or hormonally induced expression of sperm and eggs from the donors; 3) whether sperm can be stored for short periods of time; and 4) how to inseminate eggs in a Petri dish. The rest of this section will outline these basic concepts as well as additional considerations for establishing IVF protocols. Developing these techniques does not require specialized equipment and can be done by veterinarians, keepers, curators, or researchers at any institution.

### 10.1 Hormone Protocol Design

The first hurdle to overcome, prior to conducting IVF for a frog or toad, is developing a hormone regimen that will induce spermiation in males and spawning in females, despite the absence of the opposite sex. In this scenario, there are no natural cues provided by the process of mating and the hormones administered override or replace the internal chemical signals that would normally be needed during natural reproduction in the wild. Carefully designed trials should be established to understand the timing of these two events with regard to hormone administration since the success of IVF depends upon having good quality gametes that are released from both sexes within a working time frame. When first designing hormone trials for IVF the authors recommend starting with the male as they often undergo

continuous spermatogenesis with fairly short recovery periods between spermiation events. Females, on the other hand, can take several months to mature another cohort of follicles that will develop into mature eggs (Section 8 Priming Hormones).

When first designing an experiment for sperm collection, it is recommended to practice upon common toad species (e.g., American toads or Fowler toads) so that the techniques of sperm collection and evaluation are familiar to the practitioner. Bufonids are easier to practice on because of their size, ease of restraint, urination upon handling, and wide range of hormone resiliency. Practice will make the novice more comfortable with the various challenges when moving to other less common genera and smaller species.

Use the available literature to select a hormone concentration and scale down to understand the hormone's effectiveness in inducing spermiation in the species. For example, if a common concentration of LHRH for a 30 g toad is 20 µg/100 µl, perhaps test 10 µg and 5 µg as well (i.e., half the dosage each time) to see how decreasing the effective dose impacts sperm production. If no detrimental effect on the male was found at the 20 µg dose or lower, attempt a 40 µg dose to see if the animal's sperm production is improved. The goal is to acquire as much sperm as possible to deposit over the top of the eggs when conducting IVF. Sperm concentration can be evaluated using a hemacytometer, which would typically be found in a veterinary hospital or research lab. A bright field light microscope or phase contrast microscope will be needed to evaluate the sperm sample. The percent motility of a sperm sample can be evaluated by counting the number of moving sperm out of 100.

The next important factor to consider is time. Once hormones are administered to the male toad or frog, it is a good idea to begin sperm collection within 30 minutes and continue collections every hour until the sperm is depleted. Collecting sperm this frequently will provide an idea of when peak sperm production occurs for the species of interest and how often spermic urine can be obtained. For example, when conducting IVF for Fowler toads, the authors collect sperm from individual males at 3, 4, 5, 6, 7, and 8 hours post-administration (similar to Figure 14 for American toads). In this way, plenty of sperm will be banked from each male and later used to fertilize eggs. By knowing what the optimal hormone concentration is for obtaining sperm and when to begin collections (i.e., does sperm collection need to start 30 minutes post-administration of hormones as is the case for some ranids, or three hours as needed for most bufonids), one can establish a sufficient source of banked material for performing IVF in the species of interest. The more sperm available in reserve the more eggs that can be fertilized. Do not rely on one collection event to produce enough sperm for multiple IVF inseminations, as the male may not produce spermic urine at the specified time.

Sperm collection protocols should be ready prior to designing hormone protocols for females, so that one is prepared to conduct IVF in the event of successful spawning. Before attempting the priming regimen described in Section 8, begin with a single ovulatory injection of LHRH or hCG instead of a priming series, to see if the species of interest is more responsive to ovulation and spawning than the temperate animals used to develop the priming series protocol. This may minimize the amount of hormone needed and provides an indication of whether a primer is even necessary. If a single ovulatory injection does not induce spawning, the process may be repeated several weeks later, as the dose initially administered may act as a priming dose for that second treatment. Closely tracking the time between hormone treatments and spawning is helpful for designing protocols leading up to IVF. For example, knowing that the female spawns eggs 12 hours PA of hormone may make it preferable to conduct the injections late at night so that the animal will be laying eggs the next morning while staff is at work, instead of overnight if treatment is given in the morning.

Female anurans may respond to hormone treatments in one of two ways: 1) they may spawn eggs spontaneously (i.e., drop eggs on her own); or 2) they may have to be expressed from the body cavity by squeezing. There are advantages and disadvantages to each of these two responses. First, spontaneously spawning eggs into the water eliminates the need to express them, thereby reducing the risk of harm to the female through handling and manipulation. However, if eggs are spawned into the water, the person conducting the IVF must be ready immediately to remove the eggs from the water and fertilize them, as eggs left in water for more than 30 minutes can lose fertilization capacity (Figure 16). This loss in fertilization potential arises from the hardening of the egg-jelly after a short period of time thereby preventing penetration of sperm. The hardening of egg-jelly is a mechanism that has presumably evolved to serve as a natural block to *polyspermy*, fertilization of an egg by multiple sperm (Hollinger and Corton, 1980; Elinson, 1986). The risk of eggs being dropped in the water and losing fertilization potential quickly means constant vigilance by the person conducting the IVF (this is why injecting late at night may be more favorable so the IVF occurs during the next day). Second, expressing eggs has the advantage of permitting IVF to be conducted at the researcher's convenience. However, egg expression can be somewhat risky to the animal if not conducted properly and it is critical to understand the duration of a squeeze and the correct pressure to release the eggs without injuring the female.

**Figure 16**. Fowler toad eggs are spontaneously ovulated and spawned by the female 11-14 hrs PA of hormone. Through vigilance and catching the female at the exact moment of spawning, Dr. Erin Willis is able to pull the eggs gently from the cloaca of the female and immediately take them to a Petri dish for fertilization. In this scenario, it is not necessary to express eggs from the female.

### 10.2 Sperm and Egg Collection

The collection of sperm and eggs from amphibians is generally the most time-consuming step when developing successful IVF protocols. For example, collection of sperm can be as easy as holding a male over a Petri dish [e.g., *Anaxyrus (=Bufo) fowleri*) to more complicated techniques such as abdominal palpation for sperm release (e.g., *Andrias davidianus*; Figure 17), or the need for more invasive procedures such as cannulating the cloaca *[Lithobates (=Rana) pipiens]*. The following sections will outline some of the various techniques for collecting sperm and eggs from anurans that may be applicable when designing protocols for new species.

Sperm from Males

Collecting sperm from anurans can be quite simple or challenging depending on the species and size of the animal. For example, toads urinate quite readily upon handling as part of their defense mechanism and the sperm can be easily obtained through the urine, but *Ranids* require catheterization to obtain sperm. It is important to remember that in order for the male to build up a constant supply of spermic urine it must be confined to a small container (see example in Figure 18) with enough water to cover its ventral side or 'drink pouch'.

**Figure 17:** Chinese giant salamanders (*Andrias davidianus*) are being reproduced in hatcheries using both natural breeding and assisted reproductive technologies. Hormones are administered based on the individual's specific body weight; sperm or eggs can be collected by gently palpating/massaging the abdomen.

When picking up a male toad for sperm collection it is important to grasp the male quickly and securely and remove him from the container, otherwise he is likely to urinate in the holding container and the sample will be lost. Once the toad is removed from the container quickly pat his ventral side dry with a tissue to remove any water from running down his body into the Petri dish, leaving the researcher uncertain about whether or not the toad urinated, and possibly diluting the spermic urine sample. Figure 18 describes two methods for spermic urine collection, the *cradle method* and the *girdle method*.

**Figure 18.** Spermic urine collection for toads (bufonids). The **left** photo shows the *cradle method* collection of spermic urine from a male Fowler toad. The male is held over a small Petri dish and the ring finger is used to spread his legs apart to induce urination. It is important that some movement is allowed, as this often helps them to express urine as muscles contract in the legs during a struggle to escape. When first picked up, the toad's ventral abdomen was quickly dried with a tissue so that researchers are confident that all liquid collected is urine. The *girdle hold* technique for the collection of urine is shown on the **right**. A male Wyoming toad is gently held and squeezed at the waist so that his legs are forced to dangle below his body and are unable to be retracted. Some animals are stimulated by the girdle hold more than the cradle method.

The researchers have found that it is important to stop handling the animal as soon as urination occurs in order to habituate the male to urinating when being held; the reward being placed back in his enclosure, reinforcing his natural defense mechanism. If the male does not urinate quickly upon handling, a loud barking noise mimicking a predator is employed to scare the toad into urination. While somewhat comical to other people in the lab or neighboring rooms, this barking method is quite successful. Be careful not to get too close to the toad when imitating a predator as he may stream urine toward the researcher, rather than into the dish. Many a good sperm sample has been lost onto clothing.

When collecting spermic urine it is recommended to obtain the following data:

- Volume of spermic urine
- Percent motility
- Percent moving forward
- Quality of forward progression (0=no movement, while 5= fastest movement)
- Percent abnormal sperm (morphology)
- Concentration
- Viability
- Presence /absence of mitochondrial vesicle (Figure 19)

**Figure 19.** Microscope view of Fowler toad sperm. The head is long and needle shaped with a bi-flagellate tail that is connected by a thin membrane to form a ribbon-like structure. Note the large spherical mitochondrial vesicle at the posterior of the head. This structure is very prominent in bufonids but difficult to observe in ranids (Kouba et al., 2003).

While collecting sperm from toads can be quite easy, obtaining sperm from other genera can be more challenging. Many ranids, for instance, are reluctant to urinate upon handling and produce much smaller volumes of spermic urine (50-200 µl spermic urine for leopard frogs and gopher frogs versus 500-1000 µl for toad species). In addition, they are also very difficult to restrain compared to toads due to their slippery skin and mucous secretion release. Figure 20 shows a technique employed for restraining leopard frogs to collect spermic urine. In order to obtain sperm from ranids, a small (0.86 mm I.D. x 1.32 mm O.D.) plastic intravenous (IV) medical catheter tubing[8] is used that has been fire-polished at the tip using a lighter to smooth the edges. Carefully insert the catheter a short distance into the cloacal opening and gently use it to probe the bladder of the frog until urine is released into the tubing. The exact distance the catheter will be inserted depends upon the size and length of the frog; use good judgment on the depth of insertion, and do not force the tubing if there is resistance. The end of the tubing can then be placed into a small eppendorf tube and the sperm pushed out by blowing on the opposite end.

8 Scientific Commodities Inc.; catalog #BB31785-V/5; www.scicominc.com

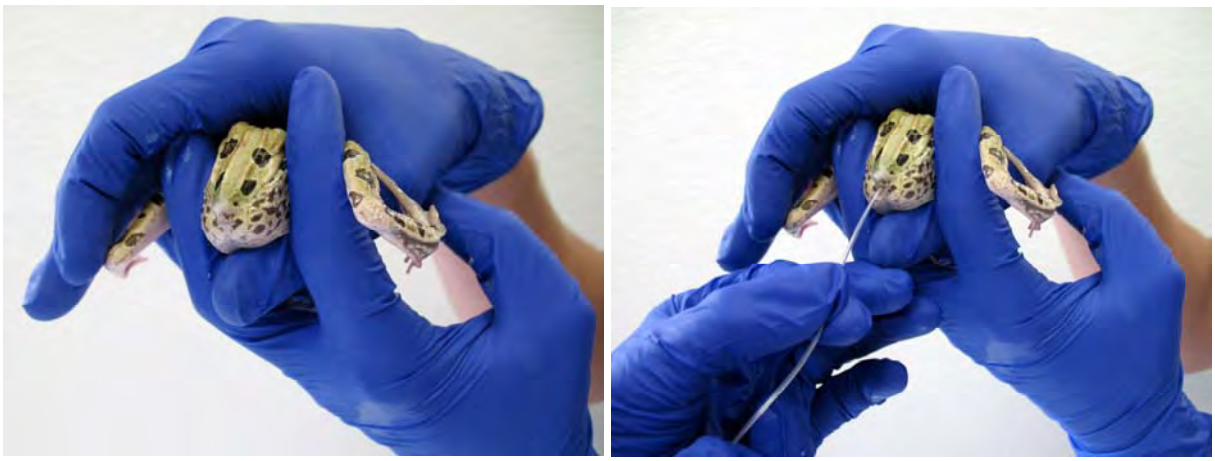

**Figure 20.** Collecting spermic urine from a leopard frog using soft medical catheter tubing. The photo on the **left** shows how to hold and restrain the leopard frog for cannulating the cloaca. Wrap a damp paper towel around the front of the animal to protect its eyes and prevent escaping forward. This hold keeps the hind legs away from the backside where they could knock the collection apparatus out of the researcher's hands. The photo on the **right** shows the cannulation procedure; note the technique requires two people: one person to restrain the frog and the other to cannulate the cloaca. The soft medical catheter tubing should be sterilized in ethanol and rinsed with sterile saline several times before use. In addition, the ends of the tubing can be rounded to prevent tearing of cloaca skin by quickly flame-polishing the end of the tubing so that it melts slightly. Know the depth trying to be reached and do not extend beyond this limit; a pen mark can be made on the tubing if necessary as an indicator for where to stop. Move the tubing slowly back and forth until urine is seen flowing into the tube. Once the urine flow stops, the fluid can be slowly dispelled into a small eppendorf tube by blowing on the clear end.

Eggs from Females

Expressing eggs from females that do not spawn spontaneously is one of the more challenging aspects of developing IVF protocols due to the inherent risk to the donor animal if squeezed too hard or for too long. It is recommended that before this is attempted on any threatened or endangered species, the method is practiced on common species to understand the limitations and levels of pressure that need to be applied to release eggs from the abdomen. It would also be prudent to be trained by someone who has performed this technique before. A good indication that eggs are in the oviduct and ready to be expressed is the presence of egg-jelly in the female's urine. A small solid inoculation probe (a plastic rod that is rounded at the end) may also be inserted into the cloaca and moved side to side to determine if there are any eggs at the opening. Do not twist this probe as it may accidentally tear skin or rectal tissue; only move side to side or up and down.

Before grasping the female, a damp paper towel should be wrapped around the animal's head to protect its eyes from any abrasions. The towel also offers additional handling security for slippery animals. The rear legs should be pulled up against the body rather than extended, and the female grasped as described in Figure 21. The inoculation probe used is inserted into the cloaca to release the pressure of the opening on the oviduct and to help express the eggs. The gentle pressure and squeezing technique is done in coordination with the movement of the probe. Only apply pressure for short periods of time (30 seconds or less), and once a strand of eggs is extruded, stop and let the female rest for 30 minutes or more before repeating. After eggs begin to drop from the female the probe is used to slightly enlarge the cloacal opening, and pressure on the abdomen may be reduced; once the eggs start flowing they tend to come out more freely.

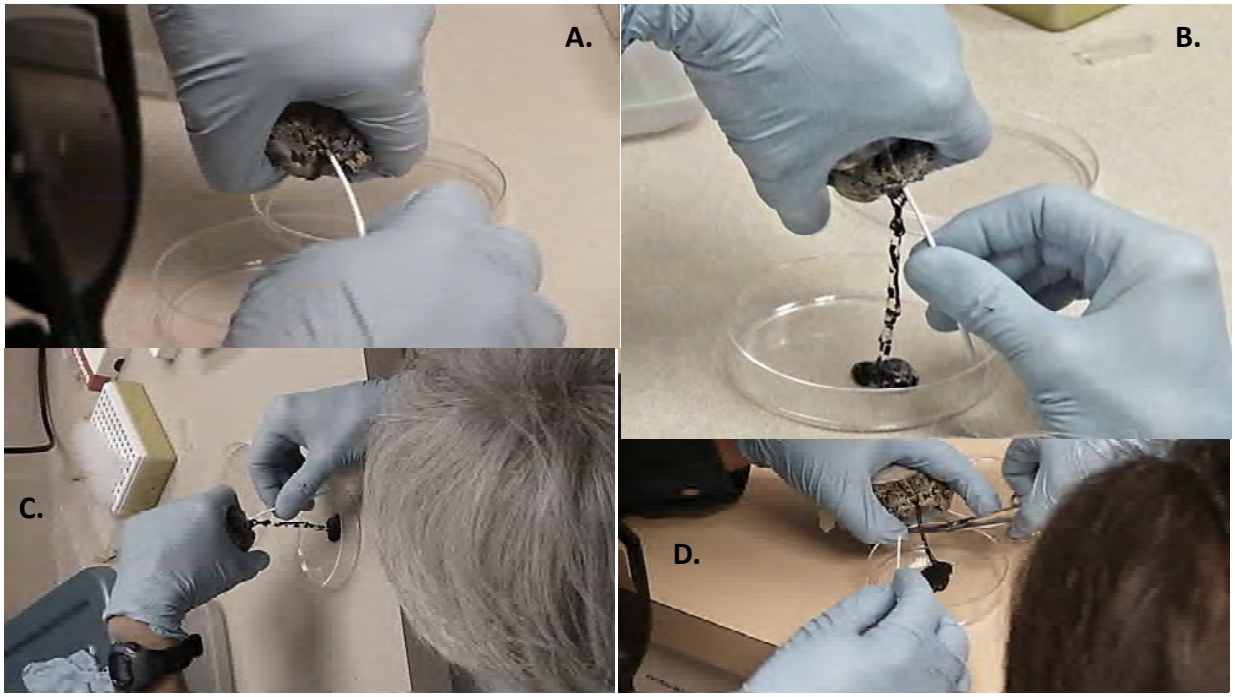


**Figure 21.** Expressing eggs from female boreal toads. **A:** An innoculation probe to release pressure from the cloaca. A damp paper towel is wrapped around the top half of the female to protect her eyes and prevent her from escaping forward. **B:** The female's legs are held in close to the body and moderate pressure is exerted on the ventral abdomen using the index and ring finger while the thumb and pointer finger exert slight pressure on the sides. In order to promote expulsion of the eggs from the cloaca a solid, plastic inoculation probe is inserted and moved back and forth to expand the cloaca opening gently (do not twist, move side to side only). **C:** The probe needs to be inserted into the animal at the same angle as its body direction. Do not angle the back end upward, as this is a common mistake which pushes the tip toward the ventral abdomen. For the female's health, avoid inserting the catheter very deeply, do not squeeze her for longer than 30 seconds, and permit her to rest 20-30 minutes between efforts to express eggs. **D:** Eggs can be cut with a pair of scissors to extract between 100-200 eggs for IVF.

### 10.3 Sperm Storage

Amphibian sperm studied to date are not very susceptible to cold shock, at least up to the point of freezing. This means that immediately upon collection, spermic urine can be placed into a refrigerator (4 C) for short-term storage. By reducing the ambient temperature of the sperm to near 4 C the metabolism of the sperm are slowed, extending their lifespan and fertilization capacity. It has been found that sperm stored in the refrigerator can last nearly two weeks although the motility and forward progression of the sperm decline over time (Figure 22). The advantage of collecting sperm samples in advance and storing them in the refrigerator is that this allows the sperm to be readily available when eggs are spawned. When working with a new species, it is advisable to see if sperm placed into the refrigerator will last several days. This provides an indication of the flexibility for timing of IVF if the female does not drop her eggs as early as planned. The authors' lab has shown that good quality Fowler toad sperm stored for eight days in the refrigerator are still able to fertilize eggs at nearly the same rate as sperm stored for just one day (unpublished results). Recently, Mississippi gopher frog sperm was collected at the Memphis Zoo, chilled, and shipped overnight to the Omaha's Henry Doorly Zoo in Nebraska for IVF. Nearly 200 gopher frog eggs were fertilized by IVF using these shipped samples from the Memphis Zoo; this achievement represents the world's first conveyance of sperm collected from live endangered animals to another institution for amphibian conservation management (Kouba et al., 2012). This significant breakthrough is proof of concept that this technology may be exportable and applicable to other institutions attempting to reproduce and genetically manage their animal collections without transporting individuals between facilities.

Short-term storage of eggs is much more challenging than that of spermatozoa. Even placing eggs into the refrigerator does not seem to slow the chemical compositional changes that egg-jelly undergoes when exposed to water. Once eggs are deposited into water there is a very short window of opportunity for fertilization. There will likely be species-specific differences in the time eggs can undergo fertilization once the egg-jelly is exposed to water, but most species studied to date suggest a window of 10-30 minutes. Some researchers have used simplified amphibian ringers (SAR) solution to extend the fertilization potential of the eggs and slow the hardening of the egg-jelly; however, this will only add hours rather than days to the window of opportunity (unpublished data). The use of SAR solution also requires the eggs to undergo numerous wash steps with water prior to fertilization because the SAR salt concentrations inhibit sperm motility and thus prevent fertilization.

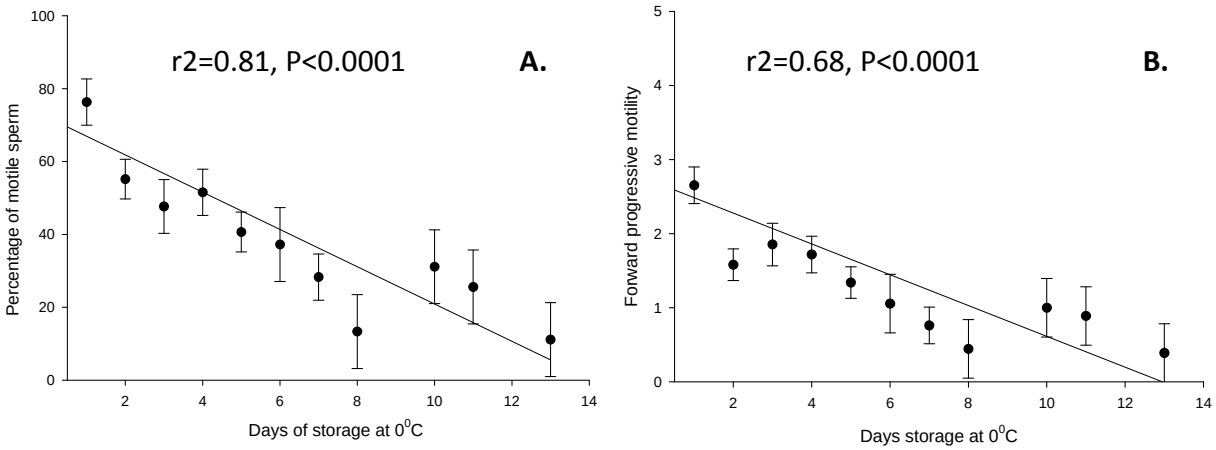


**Figure 22.** The decline in the percent motility (**A**) and forward progressive motility (**B**) of Fowler toad sperm stored at 0 C over two weeks. There was a significant correlation between storage time and percent motility or degree of forward progression. Sperm cells held at cold temperatures (e.g., 4 C) have a slowed metabolism that prolongs survivability. If spermic urine is kept at room temperature, sperm are dead within 30-120 minutes (Kouba et al., 2009).

### 10.4 In Vitro Fertilization

To conduct IVF in anurans, a procedure called *dry fertilization* is conducted by dropping sperm onto jellied eggs in a Petri dish and then allowing them to stand for five minutes without the presence of water (Hollinger and Corton, 1980). After five minutes, the eggs are slowly flooded with aerated tap water or reconstituted RO water and set aside for evaluation of cleavage rate within 4-5 hours. Figure 23 shows an outline of the three steps involved in IVF. It is important to minimize disturbing the eggs once deposited into a Petri dish for fertilization, since excess movement or prodding can cause the eggs to behave as if they have been fertilized (auto-activate); i.e., a chemical reaction will be initiated that will prevent fertilization by sperm when co-incubated. These auto-activated eggs may develop early cleavage similar to a fertilized embryo, but are not actually fertilized and are typically asymmetrical in shape.

As mentioned above, if eggs are ovulated into SAR, it will be necessary to wash them at least three times before fertilizing with sperm, as the higher osmolality (salt concentrations) in SAR inhibits sperm motility and thus, fertilization. The optimal sperm to egg ratio will be highly species-specific (e.g., some species can lay up to 8,000 eggs while others may only lay 10-20 eggs). The ratio of sperm to egg is one aspect that can limit the success of IVF protocols (Cabada, 1975; Edwards et al., 2004). In general, 100-200 eggs are fertilized with 1 x $10^5$ motile sperm/ml, or more, which seems to provide a high rate of fertilization (Kouba et al., 2009). If unable to count the concentration of sperm, the researcher should attempt to

measure the volume of spermic urine collected and divide evenly among several dishes, each containing 100-200 eggs. The minimum volume to cover the eggs can vary, but it is recommended to use 100 µl of spermic urine or more; volumes less than 50 µl can be difficult to spread across that many eggs.

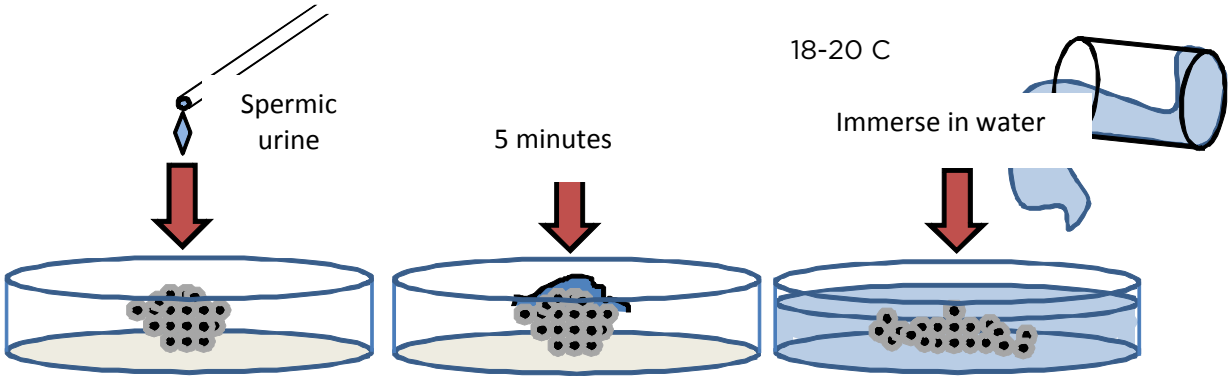


**Figure 23:** Three steps involved in the process of *dry fertilization* for anurans. 100-200 eggs are placed into a Petri dish and mixed with 100 µl or more of spermic urine (concentration >1 x $10^5$ sperm/ml) and allowed to stand. After five minutes, slowly flood the Petri dish with RO water or aged, aerated tap water. Cleavage rate can be evaluated about four hours post-fertilization.

Cleavage is very easy to identify in amphibian embryos and is visible using a stereo-microscope as early as three hours post-fertilization (Figure 24), although species may take as long as 4-5 hours. Temperature of the surrounding environment may also impact the speed of development. It is better to count cleavage rates based on the 8-cell or higher stage to ensure that all eggs have had enough time to cleave and the value recorded is an accurate representation of fertilization. However, if the researcher waits too long, the fertilized eggs will reach blastula stage and the different cells of the embryo will no longer be discernable (i.e., it will once again look like a single egg).

**Figure 24:** A Fowler toad IVF embryo at the 16-cell cleavage stage, five hours post-fertilization. There are about eight cells visible above and eight cells not visible underneath. Cleaved embryos are easily distinguishable from non-fertilized eggs before the blastula stage.

Amphibian eggs are comprised of an animal pole (dark in color) and light-colored vegetal pole (Figure 9). Most amphibian eggs, if fertilized, will rotate so that the dark animal pole faces upwards. If no stereomicroscope is available to examine them, the percentage of eggs that are fully or partially showing the vegetal pole at fertilization versus 4-5 hours later will give some indication of how successful fertilization was. The color rotation in eggs can be difficult to observe in some species that do not have a strong dichromic separation of the poles (e.g., gopher frog eggs).

There have been limited studies on IVF in endangered or threatened species yet the current technologies could be easily adapted for zoological institutions attempting to breed species that are difficult to reproduce. A perfect example of how IVF has been used to conserve a species is the production of over 2000 endangered Wyoming toads in the Memphis Zoo's laboratory by IVF (Browne et al., 2006) and over 5000 boreal toads in Colorado with the subsequent release of the tadpoles into the wild (Colorado Native Aquatic Species Restoration Facility, unpublished data). No mammalian conservation program can boast of this many threatened individuals being released into the wild from ART, highlighting the value of these technologies to amphibian species recovery.

## 11. RESOURCES

| Hormones | | Catalogue Number |
|---|---|---|
| | AVT | Sigma-Aldrich® #V0130 |
| | hCG | Sigma-Aldrich® #C1063 or #CG5 |
| | LHRH analog (GnRH) | Sigma-Aldrich® LHRHa #L4513<br><br>Animal Health International, Inc (formerly DVM Resources) #IVA022219<br><br>Argent Labs LHRHa (fish isotype) #C-LHRH-AN-1mg |
| **Websites** | | **Material** |
| | www.amphibianark.org | Science and Research Portal & Husbandry guides |
| | www.australasianzookeeping.org | Amphibian Husbandry Manuals |
| | http://tropicalis.berkeley.edu/home | *X. tropicalis*: Experimental Techniques & Protocols |
| | www.cbsg.org/cbsg/workshopreports/26/amphibian_disease_manual.pdf | Disease Control in Captive Assurance Colonies |
| | www.iucn.org | Amphibian Conservation Action Plan |
| **Books/Media** | | **Author** |
| | Amphibian Medicine and Captive Husbandry | Author: Kevin N. Wright & Brent R. Whitaker |
| | Biology of Amphibians | Author: William E. Duellman & Linda Trueb |
| | Hormone and Reproduction of Vertebrates (Volume 2: Amphibians) | Author: David O. Norris & Kristin H. Lopez |
| | Early Development of *Xenopus laevis*: A Laboratory Manual | Author: Hazel L.Sive, Robert M. Grainger & Richard M. Harland |
| | Cryopreservation in Aquatic Species (2nd Edition) | Edited by: Terrence R. Tiersch & Christopher C. Green |
| | Reproductive Biology and Phylogeny of Anura | Edited by: Barrie G.M. Jamieson |
| | Reproduction of Amphibians | Edited by: Maria Ogielska |

CONTACTS:

| Name | Email | Institution | Expertise | Country |
|---|---|---|---|---|
| Aimee Silla | Aimee.Silla@gmx.com | UNIVERSITY OF WOLLONGONG | Hormones | AUS |
| Andy Kouba | akouba@memphiszoo.org | MEMPHIS ZOO | Hormones, Cryo, IVF | USA |
| Blair Hedges | Sbh1@psu.edu | PENN STATE UNIVERSITY | Cryopreservation | USA |
| Carrie Vance | cvance@memphiszoo.org | MEMPHIS ZOO | Hormones, Cryo, IVF | USA |
| Cecilia Langhorne | cjl224@msstate.edu | MISSISSIPPI STATE UNIVERSITY | Hormones, Cryo, IVF | USA |
| Chester Figiel | chester_figiel@fws.gov | US FISH AND WILDLIFE SERVICE | Hormones, Cryo, IVF | USA |
| Frank Molinia | moliniaf@landcareresearch.co.nz | LANDCARE RESEARCH | Hormones, Cryo, IVF | NZ |
| Gina Della Togna | DellaTognaG@si.edu | SMITHSONIAN INSTITUTE | Hormones | USA |
| Helen Robertson | helen.robertson@perthzoo.wa.gov.au | PERTH ZOO | Hormones, IVF | AUS |
| Jen Germano | jgermano@sandiegozoo.org | SAN DIEGO ZOO | Hormones | USA |
| John Clulow | john.clulow@newcastle.edu.au | UNIVERSITY OF NEWCASTLE | Hormones, Cryo, IVF | AUS |
| Lucia Arregui | lucia.arregui@uam.es | UNIVERSIDAD AUTONOMA DE MADRID | Hormones, IVF | SPAIN |
| Marlys Houck | mhouck@sandiegozoo.org | SAN DIEGO ZOO | Cryo, tissue banks | USA |
| Natalie Calatayud | nec62@msstate.edu | MISSISSIPPI STATE UNIVERSITY | Hormones, IVF | USA |
| Phil Bishop | phil.bishop@iucn.org | OTAGO UNIVERSITY | Hormones | NZ |
| Rhiannon Lloyd | rhiannon.lloyd@ioz.ac.uk | INSTITUTE OF ZOOLOGY | Hormones, Cryo, IVF | UK |
| Scott Willard | swillard@bch.msstate.edu | MISSISSIPPI STATE UNIVERSITY | Hormones, IVF | USA |
| Terri Roth | terri.roth@cincinnatizoo.org | CINCINNATI ZOO | Hormones, Cryo, IVF | USA |
| Trish Rowlison | tmr207@msstate.edu | MISSISSIPPI STATE UNIVERSITY | Hormones, IVF | USA |
| Vance Trudeau | trudeauv@uOttawa.ca | UNIVERSITY OF OTTAWA | Hormones, IVF | CAN |

Legend: IVF = in vitro fertilization; Cryo = cryopreservation of amphibian sperm; NZ = New Zealand; USA = United States of American; AUS = Australia; CAN = Canada; UK = United Kingdom

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## 12. GLOSSARY

**Amplexus** – A form of pseudocopulation in which a male amphibian grasps a female with his legs as part of the mating process. At the same time, or with some time delay, the male fertilizes the female's eggs with fluid containing sperm (usually urine).

**Androgens** – The generic term for any natural or synthetic compound, usually a steroid hormone that stimulates or controls the development and maintenance of male characteristics in vertebrates by binding to androgen receptors.

**Antagonist** – A type of receptor ligand or drug that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses. Antagonist activity may be reversible or irreversible depending on the longevity of the antagonist–receptor complex, which in turn, depends on the nature of antagonist receptor binding.

**Anti-diuretic hormones** – They control the reabsorption of molecules in the tubules of the kidneys by affecting tissue permeability. These hormones also increase peripheral vascular resistance, which in turn increases arterial blood pressure. These hormones play a key role in homeostasis and the regulation of water, glucose, and salts in the blood.

**Anurans** – The taxonomic order of organisms used to classify frogs and toads.

**Arginine Vasotocin (AVT)** – A neuropeptide that modulates reproductive behavior. In females, AVT can induce phonotaxis and egg deposition. In males, AVT can induce calling, amplexus, and spermiation.

**Artificial insemination (or fertilization) (AI or AF) –** The process by which sperm is manually applied to eggs for the purpose of fertilization by using means other than sexual intercourse or natural insemination.

**Assisted Reproductive Technologies (ART) –** A general term referring to methods used to achieve pregnancy by artificial or partially-artificial means.

**Atresia** – The process in which immature ovarian follicles degenerate and are subsequently re-absorbed during the follicular phase of the estrous cycle.

**Corpus Luteum (CL)** – A temporary endocrine structure in mammals, involved in production of relatively high levels of progesterone. The main function is to maintain embryo survivorship.

**Decapeptide** – A peptide containing ten amino acids.

**Desensitization** – The reduction or elimination of an organism's reaction to the same transmitter stimulus.

**Dopamine** – A neurohormone released by the hypothalamus which acts to inhibit gonadotropin hormone secretion from the anterior pituitary.

**Domperidone** – A drug that can be used to down-regulate dopamine receptors in the brain. By doing so, the anterior pituitary can release gonadotropin hormones (such as LH and FSH).

**Dorsal lymph sac (DLS) injection** – The injection of a substance into the lymph sacs located laterally to the dorsal midline of the body.

**Down-regulation** – The process by which a cell decreases the quantity of a cellular component in response to an external variable (such as a hormone). An increase of a cellular component is *up-regulation*.

**Dystocia** – Refers to an abnormal retention of eggs or complicated parturition. Dystocia often results in the spoiling of eggs and possibly death of the female amphibian.

**Egg maturation** – The maturation of eggs to the stage of development that is capable of fertilization.

**Endocrine system** – A system of glands, each of which secretes a type of hormone directly into the bloodstream to regulate the body. The endocrine system is in contrast to the exocrine system, which secretes its chemicals using ducts.

**Estrogens** – A group of compounds named for their importance in the estrous cycle. They are the primary female sex hormones.

**Fertilization** – The fusion of gametes to produce a new organism. In animals, the process involves the fusion of an egg with a sperm, which eventually leads to the development of an embryo.

**Follicle** – The basic unit of female reproductive biology, each is composed of roughly spherical aggregations of cells found in the ovary. They contain a single immature egg. These structures are periodically initiated to grow and develop, culminating in ovulation of eggs.

**Follicle Stimulating Hormone (FSH) –** A hormone produced by the anterior pituitary gland. In females, FSH stimulates the growth and recruitment of immature ovarian follicles in the ovary. In early premature follicles, FSH is the major survival factor that rescues the follicles from cell death. In males, FSH stimulates primary spermatocytes to undergo the first division of meiosis, which then form secondary spermatocytes. It acts synergistically with LH.

**Gametogenesis** – A biological process by which diploid or haploid precursor cells undergo cell division and differentiation to form mature haploid gametes. Typically refers to spermatogenesis in males and oogenesis in females.

**Gonadotropin Releasing Hormone (GnRH)** – Also known as Luteinizing-hormone-releasing hormone (LHRH), is a peptide hormone responsible for the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. GnRH is synthesized and released from neurons within the hypothalamus.

**Gonadotropins** – Protein hormones secreted by gonadotrope cells of the pituitary gland of vertebrates. These hormones are central to the complex endocrine system that regulates normal growth, sexual development, and reproductive function.

**Hibernaculums** – Hibernation chamber.

**Homogenate** – Material that is uniform in composition or character.

**Hormone** – A chemical released by a cell or a gland in one part of the body that sends out messages that affect cells in other parts of the organism. Only a small amount of hormone is required to alter cell metabolism.

**Hypothalamus** – A portion of the brain that contains a number of small nuclei with a variety of functions. One of the most important functions of the hypothalamus is to link the nervous system to the endocrine system via the pituitary gland.

**Human Chorionic Gonadotropin (hCG)** – A human glycoprotein (protein bound to sugar chain) hormone produced during pregnancy that is made by the developing embryo after conception and later by the placenta. It can be used in other species to stimulate effects similar to the Lutenizing Hormone glycoprotein.

**Immunoassay** – An assay is a biochemical test for measuring the presence or concentration of a substance in solutions that frequently contain a complex mixture of substances. Substances in biological liquids (e.g., serum or urine) are frequently assayed using immunoassay methods. Immunoassays are based on the unique ability of an *antibody* to bind with high specificity to one or a very limited group of molecules.

**Intramuscular (IM) injection** – The injection of a substance directly into a muscle.

**Intraperitoneal (IP) injection** – The injection of a substance into the peritoneum (body cavity).

**In vitro fertilization (IVF)** – Process by which egg cells are manually fertilized by sperm outside the body.

**Lipids** – A broad group of naturally occurring molecules which includes fats, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides, phospholipids, and others. The main biological functions of lipids include energy storage, as structural components of cell membranes, and as important signaling molecules.

**Lutenizing Hormone (LH) –** A hormone produced by the anterior pituitary gland. In females, an acute rise of LH, called the *LH surge*, triggers ovulation and development of the corpus luteum (CL). In males, LH stimulates Leydig cell production of testosterone. LH acts synergistically with FSH.

**Lyophilization (or freeze-drying)** – A dehydration process typically used to preserve a perishable material or make the material more convenient for transport. Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to transition directly from the solid phase to the gas phase.

**Metaclopramide** – A drug that can be used to down-regulate dopamine receptors in the brain. By doing so, the anterior pituitary can release gonadotropin hormones (e.g., LH and FSH).

**Monoclonal antibodies** – Monospecific antibodies that are the same because they are made by identical immune cells which are all clones of a unique parent cell. Given almost any substance, it is possible to produce monoclonal antibodies that specifically bind to that substance; they can then serve to detect or purify that substance.

**Neuroendocrine signal** – Signals produced in the nervous system that enter the blood stream and act on the endocrine system.

**Neuropeptide –** Small protein-like molecule used by neurons to communicate with each other in the brain.

**Neurotransmitter –** An endogenous chemical that transmits signals from a neuron to a target cell.

**Nuptial pads** – Secondary sex characteristic present on some mature male frogs and salamanders. Triggered by androgen hormones, this breeding pad appears as a spiked epithelial swelling on the forearm that aids with grip, used primarily by males to grasp females during amplexus.

**Pimozide** – A drug that can be used to down-regulate dopamine receptors in the brain. By doing so, the anterior pituitary can release gonadotropin hormones (e.g., LH and FSH).

**Pre-vitellogenic follicles** – Follicles that have not yet reached the stage of development in which yolk formation occurs.

**Oocyte** – Female gametocyte or germ cell involved in reproduction. In other words, it is an immature ovum, or egg cell. An oocyte is produced in the ovary during female gametogenesis.

**Oviparous** – A method of reproduction involving external egg deposition, fertilization and development.

**Ovoviviparous** – A method of reproduction involving internal egg fertilization, but external development.

**Ovulation** – The process in a female's estrous cycle by which a mature ovarian follicle ruptures and discharges an ovum (also known as an oocyte or egg).

**Peptide** – Short polymers of amino acids linked by peptide bonds. They are effectively very short proteins.

**Pituitary gland** – Endocrine gland that protrudes off the bottom of the hypothalamus at the base of the brain. Its primary reproductive function is the secretion of gonadotropins (e.g., LH and FSH).

**Poikilotherms –** Organisms whose internal temperature vary considerably and are dependent on the ambient environmental temperature. They are the opposite of homeotherms (organisms which maintain thermal homeostasis, such as mammals).

**Progestin** – A synthetic progestogen that has effects similar to progesterone. Often used for corpus luteum support to maintain gestation.

**Proteolytic enzyme** – An enzyme that conducts proteolysis (i.e., begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein).

**Receptor** – A protein molecule, embedded in either the plasma or nuclear membrane or the cytoplasm of a cell, to which one or more specific kinds of signaling molecules may bind. A molecule which binds to a receptor is called a ligand, and may be a peptide or other small molecule, such as a neurotransmitter, or a hormone.

**Receptor desensitization** – The reduction or elimination of a receptor's reaction to the same neurotransmitter stimulus.

**Receptor down-regulation** – Decreasing number of cell receptors to a given hormone or neurotransmitter. This is a feedback mechanism to alter the cell's sensitivity of response.

**Spawning** – Refers to the process of releasing the eggs and sperm. Typically involves females releasing ova (unfertilized eggs) into the water, often in large quantities, while males simultaneously or sequentially release spermatozoa to fertilize the eggs.

**Spermatogenesis** – The process by which male primary germ cells undergo division, and produce a number of cells termed *spermatogonia*, from which sperm are derived.

**Sperm depletion** – Decrease of sperm output over time that can occur when males are stimulated to produce multiple ejaculates.

**Spermiation** – The process by which male sperm undergo final maturation and are secreted from the reproductive tract by urination.

**Subcutaneous (SQ) injection** – The injection of a substance into the layer of skin directly below the dermis and epidermis.

**Vitellogenesis** – The process of yolk formation via nutrients being deposited in the oocyte for supplementation of young during development of oviparous species.

**Vitellogenin** – An egg yolk precursor protein expressed in the females of nearly all oviparous species.

**Viviparous** – A method of reproduction involving internal egg fertilization and development.

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*Chapter 3*

# Hygiene and Disease Management: Field and Captivity

John Kast[1] and Nick Hanna[2]

[1]*Assistant Curator of Ectotherms, Fort Worth Zoo*
*1989 Colonial Parkway Fort Worth, TX 76110*
*jkast@fortworthzoo.org*
Photos by J. Kast

[2]*Assistant Curator of Reptiles and Amphibians, Audubon Zoo*
*P.O. BOX 4327 New Orleans, LA 70178*
*nhanna@auduboninstitute.org*

## INTRODUCTION

Amphibian populations are in decline worldwide due to a wide range of factors, both direct and indirect. Direct factors include introduced species, over-exploitation, and habitat destruction, alteration, and fragmentation, while more complex and indirect mechanisms include climate change, ultraviolet-B radiation, chemical contaminants, infectious diseases, and deformities (AmphibiaWeb, 2007). Of all the potential causes for population declines only one, *infectious disease*, has been indicated as a cause for declines in both wild and captive populations (Bradford, 1991; Daszak et al., 2001; Pessier et al., 1999; Young et al., 2007).

There are many infectious diseases in amphibians, including viruses, bacteria, water molds, fungi, and parasitic agents.[1] Several of these diseases have been documented to cause declines in the past. Red-legged disease (*Aeromonas hydrophila*) is a bacterial infection that was believed to have caused massive die-offs in the mountain yellow-legged frog (*Rana*

1 A list of sources for more information on amphibian diseases is provided at the end of this chapter.

*muscosa*) in 1979 (Bradford, 1991), while the parasitic fungus *Basidiobolus ranarum* had initially been implicated as the primary cause of extinctions of Wyoming toads (*Anaxyrus (=Bufo) baxteri*) (Taylor et al., 1999). Recently, emerging amphibian diseases such as the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*; *Bd*) and iridoviruses (*Ranavirus* spp.) have become major factors in the deaths of wild and captive amphibian populations worldwide (Mao et al., 1999; Young et al., 2007; Voigt, 2001; Zupanovic et al., 1998). Whether talking about disease transmission between captive amphibians within a single facility, between different facilities, or between wild and captive populations the conclusion is the same: *Our current husbandry practices for amphibians are no longer acceptable.* In fact, in many cases they may facilitate the spread of disease between different populations of amphibians.

Biosecurity, as defined by the US Geological Survey (USGS) National Wildlife Health Center (2007), involves three equally important aspects: 1) safety of the humans and scientists in the area; 2) decontamination/disinfection of field equipment (especially boots and nets) to prevent spread of possible infectious agent to other sites and other animal populations; and 3) careful quarantining (isolation) of live, sick animals from all other populations in the field and in laboratory colonies. This chapter is designed to reframe basic hygiene and disease management to minimize biosecurity risks in both the field and captive settings, with consideration of the evolving threats facing amphibians.

## FIELD

### Site Definition

When working in a field setting, the first precaution against the possible spread of disease among amphibian populations should be the definition of the site or sites. Defining the boundaries of a site or multiple sites can be difficult, and the mechanisms for defining borders may change from one site to the next.

The boundaries of a given site may be obvious and include natural or man-made barriers. Natural boundaries include changes in vegetation; geologic references such as mountaintops or ravines; or discrete bodies of water such as ponds, streams, wetlands, or watersheds. Individual bodies of water should each be treated as a separate site (NSW National Parks and Wildlife Service, 2001). Man-made boundaries consist of roads, development, or delineated protected wildlife areas such as state or federal refuges, parks, and reserves. Site definition is more difficult in instances where natural boundaries or man-made barriers are lacking. In these cases, the site boundary will need to be set by the researcher.

Whenever possible, plans should be made ahead of time to work in only one site per outing or have different groups working at each individual site to avoid cross-contamination. Everybody conducting fieldwork in the given site should be aware of the boundaries and how they are defined.

### On-Site Hygiene and Disease Management

When conducting fieldwork certain precautions should be taken to reduce the risk of spreading diseases. Disease can be transferred from different sites and into captive situations through several vectors including footwear, equipment, vehicles, and specimens.

Hygiene and disease management are controlled to a large extent by proper cleaning, disinfecting, and/or sterilizing. *Cleaning* involves the physical removal of organic and inorganic debris from items. Cleaning will not remove pathogens from the items, but it is a necessary step that allows the disinfecting agent to come into direct contact with pathogens on the actual surfaces of an item. Cleaning is important before disinfecting or sterilizing because most agents are inactivated by organic debris. *Disinfecting* an item by washing it

with a proper chemical agent (see Table 1) will reduce the bacteria load or pathogens to a point where they will not serve as a source of infection, but will continue to persist at low levels on the item. *Sterilization* through the use of heat, chemicals, or radiation will remove all life from an item (Wright and Whitaker, 2001).

Once an item is clean it is necessary to determine what level of sterility is acceptable. It can be difficult and time-consuming to sterilize all equipment completely in a field setting. Also, the necessary equipment to do so may not be available. Therefore, disinfecting equipment with a suitable agent for the appropriate contact time should be adequate for proper disease management in the field (see Table 1). Disinfection of items should always be done at a safe distance from bodies of water so that the solution infiltrates the soil rather than runs directly into the water. In *ex situ* settings, small items can be heat sterilized through the use of an autoclave. As an effective recommendation, heat sterilization under pressure at 160 F (71 C) for 20 minutes will eliminate both ranavirus and *Bd* (Johnson et al, 2003; Langdon, 1989).

All footwear needs to be completely disinfected before, between, and after each site is worked. Rubber boots should be worn when possible, due to the ease of cleaning and disinfecting. Canvas or leather boots/shoes are more difficult to disinfect completely and should only be worn when rubber boots are unavailable. When leaving a field site, footwear should first be cleaned of all debris and then allowed to soak in a disinfecting solution for the appropriate amount of time (see Table 1). After disinfection, footwear should be rinsed well and allowed to dry completely, taking care that the disinfecting solution does not enter any bodies of water. If necessary, having several changes of footwear available and storing used items in plastic bags between sites can be a practical alternative to immediate cleaning. Dedicated footwear should be labeled according to where they are used.

All equipment must be disinfected between sites, and if possible dedicated equipment for individual sites should be used. The use of disposable items further reduces the risk of spreading disease. Non-disposable equipment should be cleaned of all debris, soaked in any one of several disinfecting solutions (Table 1) for the appropriate amount of time, and then rinsed thoroughly, taking care to ensure that none of the disinfecting solution enters any bodies of water. If only one site is being worked, equipment can be brought back to the lab for disinfection.

Vehicles are generally less likely to be a vector for the transmission of disease than footwear and field equipment, but still should be disinfected, especially if used to cross or enter a known contaminated site. The wheels and tires should be cleaned of all debris and disinfected prior to leaving the site, using the same disinfectant that was used on footwear. Always remember to disinfect footwear before getting into a vehicle to prevent pathogens from transferring to the floor or pedals.

**Specimen Handling, Collection, and Processing**

When handling specimens in the field, even within the same site, precautions should be taken to minimize the risk of spreading pathogens. Non-powdered disposable latex or vinyl gloves are the best choice when handling specimens; however, if powdered gloves are used, they should be rinsed free of powder. A new pair of gloves should be used for each specimen. If gloves are unavailable, wash hands between specimens. Special consideration should be taken when handling tadpoles and larvae, as Cashins et al. (2008) provided some evidence that latex can be toxic to tadpoles. Therefore, tadpoles and larvae should be handled with vinyl gloves only, although it could be toxic if not rinsed with water prior to use. See Greer et al. (2009) for more information about this issue.

Latex gloves can be used for handling specimens. Use a new pair for each specimen. Vinyl gloves should be used for tadpoles and larvae.

The greatest risk for spreading disease when handling specimens occurs when animals are placed together in the same container or when containers are re-used without being disinfected first. Always use one bag or container per specimen. Do not re-use collecting bags, and utilize a new one for each specimen. Always handle specimens as little as possible. Procedures that are quick, even if potentially painful, may cause less stress than longer procedures (Speare et al., 2004). Amphibians tend not to show signs of stress immediately after handling; however, unnecessary handling should be avoided. Instruments and equipment should always be disinfected between specimens, remembering to rinse thoroughly after the appropriate amount of time. Specimens should only be released at the site of capture, and any sick or dead amphibians found should be preserved and submitted for disease diagnosis. See Chapter 4 for methods to preserve specimens for necropsy and pathology.

## CAPTIVITY

Biosecurity levels for each *ex situ species* or *species assemblage* is dependant on the ultimate goal of the program and the risk that the incoming animals pose to the existing collection and native wildlife (see Chapter 4 for more information). It is possible to achieve a realistic level of biosecurity in *ex situ* amphibian populations by following some simple and inexpensive protocols. Many of the basic hygiene principles used in the field to prevent disease transmission are applicable to the captive setting. However, additional considerations must also be addressed, including appropriate housing (e.g., permanent isolation for animals intended for reintroduction), equipment, water treatment, and staff procedures.

### Housing

Ideally, animals obtained at different sites should be housed separately from each other and from other captive animals, i.e., in permanent isolation. Separation can be done by having individual rooms per species, collection site, or by region. Again, the level of separation is dependant upon the goals of the captive program. In rooms with multiple species, animals should be housed in individual tanks, species by species. When housing different species from the same collection site or region in the same area, the assumption is made that species from the same site have already been equally exposed to any pathogens currently found at that site and can be housed together. See Chapter 5 for examples of amphibian isolation facilities.

Rooms housing amphibians should have equipment such as racks, shelves, counters, and floors that are easy to wash/mop, disinfect, and rinse. A cleaning and disinfecting schedule of all exposed surfaces is a facility's baseline defense against cross-contamination (see Table 1).

Tanks housing amphibians need to be made from materials that allow for easy cleaning and disinfecting. Non-porous materials such as glass, fiberglass, or plastic are recommended. Prior to housing any amphibians, these tanks should be cleaned, disinfected, rinsed, and thoroughly dried. The same procedure should be followed when a tank is emptied and stored.

Using automated systems for watering and draining enclosures is ideal. Not only will this decrease keeper workload but it also reduces keeper contact with enclosures and reduces the potential for disease transmission. For more information on housing amphibians and automated systems see Chapter 1.

### Equipment

Tools, protective wear, footwear, and gloves should be designated by room or enclosure to avoid any cross-contamination.

While caring for amphibians, use of proper equipment is just as important as employing proper housing types when it comes to hygiene and disease management. Equipment such as tools, gloves, footwear, and clothing should be designated for use on a room-by-room or tank-by-tank basis depending on the desired level of biosecurity.

Non-powdered disposable latex or vinyl gloves are recommended for cleaning enclosures or handling post-metamorphic animals; tadpoles and larvae should only be handled with washed vinyl gloves. If powdered gloves are used they should be rinsed free of powder prior to use. Change gloves between enclosures and store in an easily accessible location within the holding areas. Where higher levels of biosecurity are required, additional specific clothing such as surgical scrubs or Tyvek® jumpsuits (available from most laboratory or protective clothing suppliers) and dedicated footwear may need to be taken into consideration. Examples of this are discussed further Chapter 4.

Just as with gloves and clothing, any tools used while servicing enclosures should be tank- or room- specific. Tools used should be easy to clean and disinfect. Prior to disinfecting any tools they should be thoroughly cleaned to remove any organic matter, using a dual-disinfection routine (see box to right). See Table 1 for disinfecting agents, solutions, and exposure times.

**Dual-Disinfection Routine**

- Clean to remove organic matter
- Use first disinfecting agent (e.g., bleach solution)
- Rinse
- Use second disinfecting agent (e.g., ammonia solution)
- Rinse and dry

Organic cage furniture such as moss, cork bark, branching, and plants should not be recycled or transferred between enclosures. These items are difficult to disinfect and provide a perfect avenue for pathogen transferal. Non-porous cage furniture such as water bowls, plastic hide huts, and some types of rock can be put through the dual-disinfection routine and reused. See Chapter 1 for more information on enclosure furnishings.

**Water Treatment**

Water treatment in the captive setting is of utmost importance. In many cases, untreated water is the primary vector for pathogen transmission. For example, *Batrachochytrium dendrobatidis* (*Bd*), the amphibian chytrid fungus, can spread through a single drop of contaminated water (Voigt, 2001). Water treatment should occur in both incoming and outgoing water. Incoming water should be treated for standard chemical contaminants (chlorine, chloramines, etc.) through the use of carbon filtration, water additives, aeration, or reverse osmosis/deionization filtration and reconstitution (see Chapter 1 on *Water*). For captive sites within areas known to have amphibian-related diseases such as *Bd*, more extensive water treatment is necessary. Filtering water through one-micron (1μ) cartridge filters, available relatively inexpensively at hardware stores, is one method that has shown to be successful at removing chytrid spores.

For most facilities located in areas with adequate municipal sewage and water treatment systems, the risk of releasing potential pathogens into the watershed and impacting local amphibians is low. However, if managing amphibians in situations lacking access to adequate municipal water treatment, it is recommended that wastewater be transferred to central collecting tanks for chemical treatment prior to being dumped into the local watershed; helping to prevent the introduction of foreign pathogens into the environment. Before sending water to the central collecting tanks, water should be strained or mechanically filtered through the use of filter baskets, bags, floss, or other means to remove solid wastes. Large amounts of organic material will inhibit the effectiveness of disinfecting agents. Once water is collected it can be treated through the use of heat, ultraviolet sterilization, or treated with household bleach (3-6% sodium hypochlorite) to a 10% dilution. Wastewater treated with bleach should be allowed to sit for at least 24 hours prior to disposal. Treatment should be

done away from amphibian holding areas to prevent harmful side effects and possible death from chemical fumes.

**Staff Procedures**

Staff should assume that all cages are infected and that pathogens are readily transmissible between enclosures. Amphibians can be disease carriers without showing any signs of infection, thereby infecting naïve populations. Following a few simple routines on a daily basis can go a long way in preventing disease transfer.

Breeding or high-priority animals should be serviced prior to common or non-breeding specimens. Within a room or collection, service the tanks least likely to be infected first, such as long-term captives and animals that have tested negative for *Bd* or other diseases and have not exhibited any symptoms. Incoming quarantine animals should be serviced last while permanently quarantined animals should be serviced first. This is not to say the animals should be checked last in the day, but they should be serviced after the main collection to decrease the risk of transmitting a new disease into an existing *clean* (known to be uninfected) collection. See Chapter 4 for more information on staff servicing routines. Observations made early in the day can serve to spot potential problems so they can be dealt with in a timely fashion. Amphibians exhibiting any signs of disease should be dealt with immediately and any that die should have a thorough necropsy performed as soon as possible.

Taking these recommendations into account, facilities can come up with a directional service routine for their amphibian collection. For example, when servicing a room always start at the far end of the room and work towards the door, or always work in a clockwise-rotation around the room. Whatever routine is established, it should be followed in the same order every day. This way should an outbreak occur, it can be tracked and treated more effectively, hopefully with minimal loss of specimens.

## CONCLUSIONS

Following the few simple guidelines discussed in this chapter can lead to effective hygiene and disease management. In doing so, an institution can make great strides in effectively managing their amphibian programs and avoiding catastrophic consequences.

**Table 1.** Disinfection strategies suitable for killing *Batrachochytrium dendrobatidis (Bd)* and ranaviruses in field studies (Speare et al., 2004). Concentrations and times given are the minimums shown to be effective. Recommendations for *Bd* are based on Berger (2001) and Johnson et al. (2003). Recommendations for ranaviruses are based on Langdon (1989) and Miocevic et al. (1993).

| Purpose | Disinfectant | Concentration | Time | Pathogen killed |
|---|---|---|---|---|
| Disinfecting surgical equipment and other instruments (e.g., scales) | Ethanol | 70% | 1 min | *B. dendrobatidis* Ranaviruses |
| | Vircon | 1 mg/ml | 1 min | *B. dendrobatidis* Ranaviruses |
| | Benzalkonium chloride | 1 mg/ml | 1 min | *B. dendrobatidis* |
| Disinfecting collection equipment and containers | Sodium hypochlorite (bleach) | 1 % | 1 min | *B. dendrobatidis* |
| | Sodium hypochlorite (bleach) | 4 % | 15 min | Ranaviruses |
| | Didecyl dimethyl ammonium chloride | 1 in 1000 dilution | 0.5 min | *B. dendrobatidis* |
| | Complete drying | | 3 hrs or greater | *B. dendrobatidis* |
| | Heat | 140 F (60 C) | 5 min | *B. dendrobatidis* |
| | | | 15 min | Ranaviruses |
| | Heat | 98.6 F 37 C | 4 hrs | *B. dendrobatidis* |
| | Sterilizing ultraviolet light | | 1 min | Ranaviruses only |
| Disinfecting footwear | Sodium hypochlorite (bleach) | 1 % | 1 min | *B. dendrobatidis* |
| | Sodium hypochlorite (bleach) | 4 % | 15 min | Ranaviruses |
| | Didecyl dimethyl ammonium chloride | 1 in 1000 dilution | 1 min | *B. dendrobatidis* |
| | Complete drying | | 3 hrs or greater | *B. dendrobatidis* |
| Disinfecting cloth (e.g., bags, clothes) | Hot wash | 140 F (60 C) or greater | 5 min | *B. dendrobatidis* |
| | | | 15 min | Ranaviruses |

**CHECKLIST: SOURCES FOR INFORMATION ON AMPHIBIAN DISEASES**

**Amphibian Diseases Homepage**, www.jcu.edu.au/school/phtm/PHTM/frogs/ampdis.htm
Focuses on diseases of significance associated with amphibian declines.

**Amphibian Specialist Group**, www.amphibians.org
The World Conservation Union (IUCN), Species Survival Commission (SSC), Declining Amphibian Populations Task Force (DAPTF), the Global Amphibian Specialist Group (GASG) and the Global Amphibian Assessment (GAA).

**AmphibiaWeb**, www.amphibiaweb.org
Website maintained by UC Berkeley. Includes information on taxonomy and amphibian declines.

**Conservation Breeding Specialist Group**, www.cbsg.org
Download: Pessier, A.P. and J.R. Mendelson (eds.). 2010. A Manual for Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs. IUCN/SSC Conservation Breeding Specialist Group: Apple Valley, MN.

**Conservation Medicine**, www.conservationmedicine.org/amphib.htm

**Frog Web**, Amphibian Declines and Malformations, www.frogweb.nbii.gov
Center for Biological Informatics of the U.S. Geological Survey.

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*Chapter 4*

# Amphibian Quarantine and Isolation Guidelines

Shannon T. Ferrell, D.V.M., D.A.B.V.P., D.A.C.Z.M., NREMT-B

*Associate Veterinarian, Fort Worth Zoo*
*1989 Colonial Parkway Fort Worth, TX 76110-6640*
*sferrell@fortworthzoo.org*

**Caveat:** All the classifications and recommendations below were created to form a baseline of information for amphibian management decisions within AZA facilities. The recommendations represent the optimal quality of care that might not be financially or physically possible given a facility's particular limitations. Therefore, this document should not be construed as being mandated policy, but a set of suggestions that can improve amphibian care and conservation programs within participating institutions. The document can also be used to ensure that the highest recommended standards possible (such as for wastewater treatment and solid waste disposal) are incorporated into plans for new amphibian facilities. Over time, recognition of new diseases and technologies can and should be used to modify the information within this document.

## DEFINITIONS

- *Biosecurity* is the protection of the environment and its native species from foreign pathogens. In a zoo situation with display animals from different geographic locations (a cosmopolitan collection), biosecurity is applied to prevent pathogens from coming into the collection, transferring among amphibians in the collection, or moving outside the zoo into the native amphibian populations. For reintroduction programs, this concept similarly embraces all directions of disease transfer where pathogens should not move into, among, or out of assurance colonies. Pessier and Mendelson (2010) provide more detailed information about these concepts.
- *Quarantine* is the temporary medical isolation of incoming animals with resolution of any obvious or occult health problems prior to their entry into either 1) a permanent isolation facility to be used for reintroduction programs, or 2) entry into the cosmopolitan collection.
- *Best practices* are utilized as the baseline level of biosecurity methods for all captive amphibians. Amphibians housed and maintained under best practices are often in the cosmopolitan collection or under the LOW level of biosecurity (see below).
- *Permanent isolation* (PI) is when amphibians are permanently separated and protected from other captive amphibians within a cosmopolitan zoo or other amphibian collection. The goal of permanent isolation is the eventual reintroduction of these isolated animals or their progeny into the wild.

**Levels Of Biosecurity Risk for Breeding/Reintroduction Programs**
(Pessier and Mendelson, 2010)

LOW - The lowest risk situation for introduction of a significant infectious disease to wild amphibian populations occurs when survival assurance populations are located within the native country of the species or species assemblage (i.e., an amphibian faunal group that naturally occurs together in a country), and the facility houses only amphibians from inside the native country.

MODERATE - A moderate risk situation for introduction of a significant infectious disease to wild amphibian populations occurs when the captive assurance population could be exposed to amphibians that originate from outside the native range of the species.

HIGH - A high risk situation for introduction of a significant infectious disease to wild amphibian populations as the result of a reintroduction program occurs when the survival assurance population is exposed to amphibians from outside the native range of the species or is exposed to animals with infectious diseases not already present in the captive population. High risk situations occur when the captive population is held outside of appropriate biosecurity for any length of time. In general, amphibians that have been held in situations without appropriate biosecurity practices are not suitable for use in a reintroduction program. Exceptions are considered if the species is extinct in the wild, and captive populations housed under appropriate biosecurity conditions do not exist elsewhere. In these situations, a very thorough, expensive, and extended (months to years) disease risk assessment and screening procedure is necessary before consideration can be given to reintroduce these animals to the wild.

## FACILITIES

### Natural history of animal

Prior to the development of a species collection plan and construction of any amphibian facility/room, it is important to be familiar with the natural history of the species in question. Knowledge of the temperature, humidity, and light requirements with additional attention given to behavioral temperament can and should heavily influence the construction of the facility. Many species require specific water qualities and temperatures for optimal feeding and breeding that place heavy demands on construction and utilities, and that require advanced planning and budgeting. See Chapter 1 for more information.

### Location of an Amphibian Quarantine or PI facility

- *Preferred* standard
  The facility is a completely separate building from the cosmopolitan animal collection. Only a single species or species assemblage is permitted per room. Ideally, all animals in a single room should have the same ultimate role and should have a history of being maintained at a level of biosecurity appropriate for that role. Facilities that house individual species or species assemblages in self-contained units, e.g., modified shipping containers (Amphibian Research Centre, 2007), may have advantages over a single dedicated building. Note that a quarantine facility should be separated and isolated from the permanent isolation facility, but both facilities are similarly conceived and managed. This standard is most appropriate for reintroduction programs housing animals in MODERATE or HIGH risk biosecurity situations, as described above.
- *Minimum* standard
  Dedicated space in a cosmopolitan animal facility should consist of isolated rooms containing only a single species or species assemblage. It is important for managers to understand that these rooms constitute the *Amphibian Quarantine or Isolation Facility* and the removal of dedicated footwear and clothing should occur **PRIOR** to handling any

other animals. This housing situation is most appropriate for reintroduction programs with a LOW risk biosecurity rating.

**Rooms**

- Surfaces
  Walls, floors, and ceilings should be impervious to fluids, creating easier cleaning and enhancing sanitation.
- Electrical
  Water is often splashed around during cleaning of aquatic amphibians such that all electrical outlets should have ground fault circuit interrupters (GFCI).
- Environmental controls (For more information on following topics, see Chapter 1)
  - *Temperature:* Rooms need to be capable of adjusting temperatures to meet the natural historical ranges for the species and be capable of independent variation within a facility such that each room can run at a separate temperature. Temperatures within a room should ideally be warmer during the day with a small nocturnal decrease to simulate environmental fluctuations.
  - *Humidity:* Humidity can be increased by the use of free-standing humidifiers, misting systems, or changing enclosure design to optimize humidity. Non-aquatic amphibians usually need high humidity that can be provided by using a moss substrate to keep the cage environment at an optimal humidity level.
  - *Light:* Rooms and enclosures should be capable of independent light levels based on the required light cycles (most amphibians require at least 8-12 hours of light daily). Full-spectrum lighting is recommended to provide ultraviolet-B (UVB) and ultraviolet-A (UVA).

**Enclosures**

Glass, fiberglass, or plastic tanks can be used. Acceptable plastics are those used for human food storage as other industrial plastic sources can leach toxicants into the water. Plastic food storage bins (5-15 gallon/19-57 L) with custom-fabricated, ventilated lids are used frequently. Tank dimensions vary with size and number of animals housed, and can be plumbed for constant water flow and drainage, if needed (see *Water* section below for information on plumbing). Opaque containers and the use of hiding sites (PVC pipe, ceramic tiles, terra cotta pots, etc.) decrease stress and enhance welfare. Cages placed on racks at a tilt promote drainage and hygiene, maximize storage, and improve access through the lid on top. As many species can escape by climbing or jumping out of the enclosure, lids should be well-fitted and securable. For more information on enclosures, see Chapter 1.

**Water**

- Desired types
  - *Disease-free water*: Water acquired from sources determined to be free of amphibian-related diseases.
  - *Treated water*: Water treated to safeguard inhabitants against disease transmission.
    - Background information:
      - For disinfection of water by heat (either wastewater leaving the facility or incoming water), relatively low temperatures of 60 C (140 F) for 15–20 minutes are effective in eliminating the *Batrachochytrium dendrobatidis* (*Bd*, the amphibian chytrid fungus) and the EHNV *Ranavirus* in a laboratory setting (Johnson et al., 2003; Langdon, 1989). These temperatures may not be effective for bacterial spores or all viruses. If sterilization of water is desired, higher temperatures over the boiling point at 100 C (212 F) is necessary. Heating under pressure (e.g., autoclave or pressure cooker) increases the effectiveness.

        - Filtering water through one micron (1 µm) cartridge filters, available relatively inexpensively at hardware stores, is one method that may be successful at removing the zoospores (3-5 µm) of the amphibian chytrid fungus. Water filtration is not effective at removing viruses.
        - Ultraviolet light may be effective at removing ranaviruses from water, but efficacy against the amphibian chytrid fungus is questionable.
    - Heat disinfection to 60 C (140 F) for 15-20 minutes under pressure is the *preferred* method.
    - Sediment removing mechanical pre-filters with chemical treatments (i.e., chlorine or chloramines) is the *minimal* method. Improper use of chlorine agents could potentially lead to accidental and catastrophic fatal exposure for resident animals and is also of environmental concern. Aeration of water can be used to remove some chlorine compounds. Other agents (sodium thiosulfate, AmQuel®+, and/or activated charcoal) can be added to chemically-treated water to remove any chlorine compounds. If using sodium thiosulfate to remove chloramines, the water will need further treatment to remove the ammonia (i.e., zeolite or biological filter).
- Sources
    - *City/well*
      Inexpensive and commonly used. Tap water from municipalities contains lethal levels of chlorine or chloramines that should be removed by 24-48 hour aeration, chemical treatments (sodium thiosulfate or AmQuel®+), and/or activated charcoal filtration. Activated charcoal is much more effective at removal of chloramines than aeration. Well-water and tap water might have trace toxic chemicals that could be lethal to amphibians, making the use of activated charcoal preferred for treatment. Both tap water and well-water should have the pH and other water quality levels checked to ensure they are within the parameters appropriate for the species maintained. Some water sources will need to have the pH manipulated with chemical additives or buffers to be suitable for use with some amphibian species.
    - *Bottled*: Distilled or reverse-osmosis (RO) treated
      Expensive for a large-scale operation; distilled water and RO water are usually not electrolyte-balanced and can be fatal to amphibians without rebalancing with buffers, electrolytes, and pH adjustments.
    - *In-house reverse–osmosis (RO) treated*
      Expensive for a large-scale operation, but provides the highest water purity available. Only moderate volumes are generated at any given time, involving daily production by staff. This method also requires rebalancing and buffering with salts and electrolytes for safe, long-term use with amphibians.
- Plumbing/flow system types
    - *Static*: Closed systems with standing water (*dump and fill*)
      Works well for large or small groups. Enclosures should be plumbed for convenient draining and refilling purposes. These systems require daily manual labor to clean and maintain adequate water quality in the confined environment.
    - *Recirculating systems*: Closed systems
      Pumps force the water through mechanical (i.e., sand and/or charcoal) and biological filters to remove debris and nitrogenous wastes, respectively, from enclosures. Filters can become overwhelmed by debris and waste if used for large populations. These systems require regular maintenance and monitoring to ensure adequate water quality and flow rate.
    - *Continuous flow systems*: Open systems
      A constant stream of water into and out of enclosure usually by a hose, misting system, or other drip source dilutes waste to a non-toxic level in the enclosure water, and removes wastewater and debris continually. A standpipe can be employed to regulate pool depth as well as to drain water from this system. Influent water

temperature and quality needs to be regulated and treated to ensure no introduction of chlorine compounds or other toxins. Constant monitoring is necessary to prevent temperature fluctuation into extreme ranges or overflow from a blocked drain.

- Water quality testing
  Testing should be performed weekly in a quarantine facility and at least monthly in a PI facility. Accurate testing equipment is required, and staff should be trained in its correct use. Electronic colorimetric equipment[1] is highly accurate and should be considered, but is also expensive. Less expensive chemical titration kits and dip-strip tests are available and suitable for non-routine testing; however, they are less accurate and precise than electronic colorimeters. Water quality parameters to be monitored include ammonia, nitrite, nitrate, pH, phosphate, and copper, especially for totally aquatic amphibian species.
- Modification
  Water chemistries can be manipulated to enhance tadpole growth, breeding, etc. Formulas are available that detail what additives and amounts to add to tank water as needed (Wright and Whitaker, 2001).
- Disposal
  See *Sanitation* section that follows.

For more information on water, see Chapter 1.

## HUSBANDRY

### Identification

- Morphological identification
  Includes the use of physical characteristics such as size, coloration patterns, sexual dimorphism (e.g., nuptial pads in the males, toe-pad width, etc.), and/or other distinguishing markings to identify individuals within a collection. Photo-documentation is a very valuable tool, but juveniles of some species change dramatically as they age.
- External identification
  - *Toe clips*
    This inexpensive option for marking individuals involves surgical amputation of the end of specific digits based on a coding scheme for marking purposes (Donnelly et al., 1994). The tissue removed can be saved for DNA banking, the amphibian chytrid fungus polymerase chain reaction (PCR), and/or other disease investigations, if stored correctly.
  - *Attached tags or beads*
    Loose colored wire or elastic bands have been placed around the waist of frogs. Plastic, colored beads have been sewn to the limbs of amphibians using a non-absorbable suture material that passes through a muscle mass and anchors the beads permanently. Placement on animal, added weight, and potential for catching on enclosure furnishings should be taken into consideration for this method.
  - *Ink and branding*
    Traditional tattooing and branding (heat or freeze) have been used to mark amphibians successfully (Kaplan, 1959; Clarke, 1971; Daugherty, 1976). However, application of these methods varies between species and testing should be performed before it is used widely. Select a dye or method that will contrast with skin pigmentation and remain legible over time.
  - *Radiofrequency biocompatible ink*
    This special ink tattoo emits an identification-signal specific to that animal and can be read with radiofrequency.[2] This is new technology and is unknown for use in amphibians.

1 Such as the Hach® DR/890 Colorimeter
2 Somark Innovations, Inc., Saint Louis, MO – available in visible or invisible dyes

- Internal identification
  - *Microchip Identification Devices* (PIT tags)
    Subcutaneously implanted microchips function at different frequencies and levels of encryption. Some companies' microchip readers can recognize and/or identify multiple frequencies, but most only read their own frequency. ISO frequency (134.2 kHz, 15-digit numeric identity code) is becoming the world standard, and most U.S. distributers are starting to carry the ISO frequency chips and readers.[3] Surgical glue is recommended to close the implant site.
  - *Injectable elastomers*
    Phosphorescent elastomers are injected underneath the skin or into the muscle superficially (Visible Implant Elastomer or *VIE* Tags).[4] Multiple colors are available, including invisible elastomers that utilize a black light for detection. There are similar pre-cured elastomer tags with individual alphanumeric codes printed on one side (*VI Alpha*). Implanted markers may migrate.
  - *Coded Wire Tags* (CWT)
    An implanted short length of thin magnetized stainless steel wire is marked with rows of coded numbers that can be read under magnification after the wire is surgically removed from the animal.[4] Implanted markers may migrate.

Routine order

Animals in the *Amphibian PI Facility* should be processed first in the daily work schedule PRIOR to handling any animals in the cosmopolitan collection or quarantined animals. In addition, new animals brought into quarantine that are headed for permanent isolation should be cared for PRIOR to handling any cosmopolitan animals or the other quarantine amphibians. Finally, the animals in the cosmopolitan collections are serviced to PRIOR to care of any eventual cosmopolitan animals in the incoming quarantine. Summarily, the ideal final flow of contact for routine husbandry is as follows:

PI → Q animals slated for PI → cosmopolitan animals → Q animals slated for the cosmopolitan collection

**Nutrition**

- Complete, balanced diet
  Food items offered to amphibians in captive facilities are a potential source for introduction of pathogens to a population. The extent to which food items could be a source of amphibian pathogens is unknown. Most likely food items act as a mechanical vector for disease transmission (e.g., wild caught insects or aquatic invertebrates used as food are wet, and transfer water with infective zoospores of the chytrid fungus into the captive facility), rather than becoming infected with these pathogens and serving as a long term source of infection for the amphibian population (Rowley et al., 2007). A clean, reliable, and trusted source of food items is desirable for all facilities that keep captive amphibians (Pessier and Mendelson, 2010).
  - *Prey in general* – Most amphibians will attempt to eat prey items only if they are alive and moving. Prey items need to be the correct size, or they will not trigger a feeding response. When possible, offer a varied diet to provide a wider range of nutrients and better simulate a natural diet.
  - *Insects* – crickets, fruit flies, mealworms, wax moth larvae, springtails, roaches, field-sweepings, etc.
  - *Other invertebrates* – worms or crayfish
  - *Fish* – small minnows, goldfish, shiners, etc.

3 AVID® (125 kHz); Banfield® (125 and 134.2 kHz); Biomark®/Destron Fearing™ (125 and 134.2 kHz); and Trovan® (128 and 134.2 kHz)
4 Available from Northwest Marine Technology, Inc.

  - *Small animals* – rodents, lizards, amphibians, birds, or commercially-available sausages[5]
- Supplementation
  Most insects will need to be dusted with a formulated vitamin supplement to ensure proper calcium to phosphorus ratio (Ca:P) in the diet and also provision of certain vitamins, such as vitamin A.
- Feeding schedule
  Varies on needs of animals, but is usually daily for small insectivores and less frequently for larger amphibians (every other or third day). Obesity can be an issue, especially with large terrestrial amphibians, so frequency for offering large meals may range from weekly to monthly; offering smaller live insects between large meals will encourage exercise.
- Presentation/removal
  Ideally, prey items should be fresh and moving. If prey items are not consumed within 24 hours, they should be removed to keep from fouling the environment and possible reverse-predation on the amphibian. Insects such as crickets need a food source (small dish with cricket diet or rodent chow) within the amphibian's tank to keep them from attacking the amphibian.

See Chapter 1 for more information on amphibian diets.

**Sanitation**

- Cleaning schedule: *Minimum* standard with frequencies increasing as amphibian biomass and feedings increase
  - Water change frequency is dependent on the natural history of the animal and type of system used. A continuous, low-volume flow with overflow drains is preferred over the static (*dump and fill*) method and reduces stress to the animals. If closed systems are to be used, weekly or more frequent water changes are recommended, depending on whether a filtration system is employed. It is advisable to perform a water change two hours post-feeding for aquatic amphibians.
  - Daily spot cleaning by removing visible feces and unconsumed food items is effective for reducing the need for frequent complete cleaning or breakdown of enclosures. To reduce potential buildup of organic wastes and of certain pathogens, such as the rhabditiform nematodes, disposable substrates like paper towels or paper pulp should be changed frequently (every day or every other day). Reusable substrates that are easily disinfected, such as untreated foam rubber or Astroturf®, may also need to be cleaned each day. Duplicate sets of these items for each enclosure will simplify the task. The soiled set can be chemically disinfected, rinsed, dried, and ready for use the following day.
  - Complete cage break down and cleaning should be performed weekly in quarantine and at least biannually in permanent isolation or the cosmopolitan collections.
  - Attempt to clean cages at same time of day and in the same directional order to control disease spread.
- Clothing, gloves, and uniform standards
  - *Preferred* standard for working between species or species assemblages in quarantine or PI facilities:
    Dedicated clothing and footwear should be available for each species or species assemblage and changed before working with a different group. Disposable protective clothing (e.g., Tyvek® jumpsuits) may be useful in this regard. Ideally, keepers would have appropriate amenities to shower between servicing each species or species assemblage housed in the *Amphibian Quarantine or PI Facility*. Gloves should be worn while accessing amphibian enclosures, and dedicated glove use may be required per individual container, per species, or per faunal group depending on pathogen risk.

5 Natural Balance® Reptile Diet sausages

Frequent washing of the hands and arms (up to the elbows) with a disinfectant soap is recommended for husbandry staff members as a standard feature. It is especially important to wash the hands and arms before entering each room or facility and between working the different enclosures. Most appropriate for MODERATE/HIGH risk biosecurity reintroduction programs.

- *Minimum* standard for working between species or species assemblages in quarantine or PI facilities:
  Exactly same requirements as the preferred standard, but no need for showering facilities or dedicated footwear/clothing for individual rooms, instead requires dedicated footwear/clothing for the building only. Most appropriate for reintroduction programs with LOW biosecurity risk rating and/or amphibians in the cosmopolitan collection.

- Tools
  Ideally, each species or species assemblage will have its own set of tools (nets, forceps, suction tubing, scrub brushes, sponges, etc.) that will not move between cages/rooms. If tools will be used in multiple cages within a room, it is advisable that the tools be soaked in a disinfecting solution for at least 15 minutes. Tools may need to be soaked in specific or multiple disinfectants prior to use depending upon the pathogens to be eliminated (See Chapter 3 for recommendations). After each disinfectant, all tools need to be thoroughly rinsed with fresh water.
- Substrate change frequency
  For substrates that cannot be easily disinfected (e.g., organic matter and paper towels), complete replacement should be performed daily or weekly in quarantine and at least biannually in animals in PI or the cosmopolitan collection. Some substrates can be disinfected and then reused in the enclosure. Organic substrates like sphagnum moss, peat, coconut husk, soil, as well as stone and ceramic substrates like pea gravel, river rock, sand, and LECA (light weight expanded clay aggregate) can be disinfected or sterilized using heat treatment prior to use in an enclosure. A good review of this topic can be found in Pottorff (2010).
- Wastewater disposal
  For facilities that house amphibians from outside the native range (e.g., Panamanian amphibians kept in the United States) or which conduct research with amphibian pathogens, wastewater biosecurity measures are needed. Facility wastewater should be treated to minimize the risk of exporting foreign pathogens out of the facility and introducing them into the surrounding area (Brown et al., 2007). For most facilities located in areas with adequate municipal sewage and water treatment systems, the risk of releasing potential pathogens into the watershed and impacting local amphibians is low. If necessary, additional wastewater treatment from an amphibian facility can be accomplished by application of heat, ozone, or chlorine bleach (Schuur, 2003; Robertson et al., 2008). The treatment of wastewater may be incorporated into a keeper's daily schedule, such that the wastewater is collected, treated, and kept overnight before discharged. Facilities which only keep amphibians from inside the native range (e.g., southern Japanese amphibians kept in southern Japan) usually do not need to consider wastewater biosecurity measures (Pessier and Mendelson, 2010). For more information, guidelines for aquaculture facilities have been developed by the World Organisation for Animal Health (OIE, 2011).
    - Heat disinfection to 60 C (140 F) for 15-20 minutes under pressure is the *preferred* method and will kill both *Bd* zoosporangia and *Ranavirus*. At minimum, chlorine treatment of wastewater with standard household bleach added to the wastewater should take place in an amphibian-safe manner (i.e., ventilation of chemical fumes and disposal into the sewer system rather than a local watershed). Water disinfection systems should be designed to minimize introduction of toxic chemical contaminants (chlorine or chloramines) if wastewater is discharged

directly to local environments. For instance, chlorine can be neutralized by treatment with sodium thiosulfate (Browne et al., 2007) or by exposure to ultra-violet (UV) radiation (Robertson et al., 2008).

- Solid waste disposal
  Disposal of solid waste from quarantine and PI facilities (and the cosmopolitan collection in the case of a known pathogen outbreak), including all substrate, props, gloves, etc., should be decontaminated by way of incineration or heating to a minimum of 60 C (140 F) for 15-20 minutes prior to being discarded. Disposal by a medical waste hauler is an alternative.
- Carcass disposal
  For carcass disposal, institutions should follow appropriate necropsy procedures. Accepted final tissue disposal options include: incineration, alkaline tissue digestion, formalin or alcohol fixation, or disposal by a certified medical waste hauler.
- Vermin control
  Vermin in a facility can act as transport hosts for viral, bacterial, and parasitic agents. The use of mechanical trapping methods is preferred over chemical agents as many of the chemical agents (whether sprayed or stored as bait) can adversely affect amphibian health through direct toxic effects or by functioning as endocrine disruptors.
- Disinfectants
  There are no ideal disinfectants that combine wide efficacy against a variety of pathogens, low toxicity, ease of use and disposal, and low cost. A disinfectant should be carefully chosen based on all relevant factors. Reading the product label is highly recommended in order to use and dispose of the disinfectant compound(s) correctly. Equipment, cages, and surfaces should be cleaned of debris and rinsed prior to the application of any disinfectant. Prior manual removal of debris greatly enhances the efficacy of the applied disinfectant. The following disinfection methods and contact times have been recommended for amphibian settings:
    - 3-4% sodium hypochlorite (household bleach) for 1 minute on wet surfaces for *Ranavirus/Bd*
    - 70% ethanol or 1 mg/ml benzalkonium chloride for 1 minute for *Bd*
    - Desiccation or exposure to 60 C (140 F) heat for 15-20 minutes for *Ranavirus/Bd*

  Rinse all equipment, cages, and surfaces with fresh water after applying a disinfectant (see Chapter 3 for more information on hygiene and disinfection recommendations).

**QUARANTINE DURATION**

- *Preferred* standard
  All animals enter into a facility at the same time and leave at the same time (*all in - all out*). Sixty to ninety days are usually needed to detect and treat fully for pathogens, prior to release from a quarantine area. The duration might be extended depending on clinical findings. Animals will not be released from quarantine if mortalities occur from unidentified, unknown causes. If possible and practical, treatment on surviving animals should be initiated. No animals should be released from quarantine until all mortalities have stopped; disease issues are completely eliminated; and the remainder of animals are feeding, defecating, and appear healthy. This level of quarantine duration is appropriate for reintroduction animals in MODERATE to HIGH risk biosecurity situations.
- *Minimum* standard
  Exactly the same requirements as the preferred standard however, thirty days is the minimum quarantine period for LOW risk situations with little chance of incoming animals harboring virulent pathogens.

## MEDICAL CARE

Medical records

A medical record is a systematic documentation of the medical history and care given to an individual animal or group. Maintaining detailed medical records provides a basis for assessing the effectiveness of quarantine protocols, discovery of proper husbandry methods, and allowing for the analyses of the causes of morbidity and mortality in quarantine. The medical record should contain the following components:

- **Identification/inventory report.** When possible, the identification/inventory section of the medical record follows an International Species Inventory System (ISIS) format containing the following when possible: animal common name(s), taxonomic name (genus and species), sex, birth type, birth location, birth date, date of acquisition, source/identification of sire and dam, individual institution accession number, and any other identifiers such as studbook numbers, microchips, and bands.
- **Keeper notes.** All animals, whether as individuals or groups, should have some daily notation in the record made as to presence or absence of at least the following factors: feces, urine, food intake, normal behavior, and attitude. Monitor body weights weekly while animals are in quarantine and monthly for animals in PI, if practical.
- **Clinical notes by veterinary medical staff.** This section includes notes and observations on clinical diagnoses, treatments, anesthesia, and medication prescriptions.
- **Laboratory results.** Laboratory results from parasitology, serology/molecular diagnostics, microbiology, and necropsy/histopathology.

### Parasites

- Fecal parasite screening is performed the first week of quarantine and again one week before the end of quarantine. Increased frequency of screening is determined by the goals of monitoring and needs of the animals in quarantine. Animals in permanent isolation should be tested biannually, if not more often dependent on monitoring goals.
- Although many amphibians carry a commensal load of enteric flagellates that do not usually require treatment, the decision to treat will be dependent upon the parasite genus, load level, anti-parasitic agents, species temperament, and ultimate disposition plan. Trying to remove all enteric and systemic parasites via chemotherapeutics can stress the animals, change their enteric biota, and result in the animal's death. A veterinarian and amphibian manager should make a cost/benefit analysis prior to parasite treatments.
- Available medications include fenbendazole, ivermectin, and levamisole. Dosages and route can vary depending on parasite and host species (Wright and Whitaker, 2001).

### Medical diagnostics

- Physical examinations by a veterinarian familiar with amphibians
  - *Visual exam and palpation performed at least once in quarantine and PI.*
  - *Morphometrics:* Record weight and identifying markings.
  - *Clinical:* Document behavior and physical abnormalities.
- *Batrachochytrium dendrobatidis* (*Bd*; the amphibian chytrid fungus) screening via DNA probe
  - *Testing:* Perform prior to any treatments. Test at least once in quarantine and permanent isolation if the animals are from collections with a known medical history and no recently identified *Bd* infections (within one year). If the amphibians are from unknown sources or those with recent *Bd* infections, it is recommended to test at least twice to three times prior to release. Pooling of samples can be done due to logistical or financial issues, but disadvantages do exist with pooled samples.
  - *Suggested labs, cost, and collection method:* **Pisces Molecular LLC**, 2200 Central Avenue, Suite F, Boulder, CO 80301-2841, 303-546-9400; approx. $30/sample; Submit skin surface swab or scrape placed into 70% alcohol (contact Pisces for details). **Amphibian Disease Laboratory, San Diego Zoo Institute for Conservation Research**,

Amphibianlab@sandiegozoo.org or www.sandiegozooglobal.org/News/Amphibian_Disease_Laboratory/, 619-231-1515, Ext 4510; approx. $20/sample; Submit air-dried skin surface swab (contact laboratory for sample collection supplies and submission instructions).

- *Ranavirus* screening via DNA probe
  - *Testing:* There is no validated PCR assay to screen healthy animals for subclinical infections. *Ranavirus* biology is complex and still being elucidated. Samples can be collected from clinically ill amphibians using oral swabs, cloacal swabs, blood, or tissue samples for PCR. Current recommendations are to sacrifice clinically ill animals of any quarantine or PI status for disease surveillance. Tissues would be collected for histopathology and *Ranavirus* PCR on liver, kidney, and skin. If clinically ill animals cannot be sacrificed, oral and cloacal swabs can be performed, but test results may not be highly reliable.
  - *Suggested labs, cost, and collection method:* **University of Florida**, 2015 SW 16th Ave, Building 1017 Room V2-238, Gainesville, FL 32608, Phone 352-392-4700 x5775; $100/sample; Submit swab or tissue (suggested sample for living animal is cloacal swab). **Amphibian Disease Laboratory, San Diego Zoo Institute for Conservation Research**, Amphibianlab@sandiegozoo.org or www.sandiegozooglobal.org/News/Amphibian_Disease_Laboratory/, 619-231-1515, Ext 4510; approx. $25/sample (contact laboratory for sample collection supplies and instructions).
- Hematology/biochemistry

  Dependent upon the specimen's size, it is safe to collect up to 1% of body weight from a healthy animal. It is recommended not to collect blood from specimens weighing below 50 g due to safety concerns. Correct use of tricaine methanesulfonate (MS-222) can make blood collection easier with reduced stress and adverse problems. Only a veterinarian or trained individual should perform anesthesia, as mortalities can occur.
  - *Testing:* Perform at least one full blood panel in quarantine and PI animals.
  - *Suggested lab and cost:* Employ **any veterinary diagnostic laboratory** that runs reptile samples. Few normal panel values currently exist for most amphibian species in the ISIS database, making interpretation of results somewhat difficult. Based on diagnostic needs, the laboratory may have to design a complete hematology and biochemistry panel, but if limited by cost, then apply those existing for reptile species. As more amphibian-specific panels are designed and submitted to the ISIS database, the diagnostic value of any result increases for the population, improving amphibian healthcare overall.
- Necropsy

  All animals receive gross necropsies upon death with a report generated for the medical record. Necropsies should be performed by a veterinarian or trained individual to maximize diagnostic information. Bodies should be immediately refrigerated if there is any delay to the necropsy being performed. The carcass can be stored under refrigeration 2–8 C (36–46 F) for up to 24–72 hours. Do not freeze the carcass prior to necropsy. If a significant delay will occur prior to necropsy by a veterinarian or trained individual, make an incision into the coelomic cavity and immerse the entire carcass in 10% buffered formalin. Animals that are autolyzed and/or desiccated are of little diagnostic value as tissues degrade quickly. Submit recent history and water quality along with the body. Pessier and Mendelson (2010) provide an excellent resource for performing necropsies and sample collection.
  - *Sample collection for histopathology:* Samples from a fresh animal are ideal. Samples should be placed into 10% buffered formalin. Small animals (less than 10-20 g) can be placed intact into formalin if a small incision is made into the coelom to allow formalin to permeate the body cavity. Larger animals should have tissues collected by a veterinarian or trained individual. It is suggested that portions of the liver be routinely

frozen and saved from all necropsies. If multiple animals die from a disease outbreak at the same time, freeze half of the specimens at –70 C (-94 F) for future ancillary diagnostic tests, and perform necropsies and histopathology on the remaining deceased animals. Tissues will then be forwarded onto a pathologist familiar with amphibian diseases. The pathologist will generate a report for the medical record that is then used to make management decisions.

- *Sample collection for additional diagnostics:*
  - Collect skin sample for *Bd* testing (see *Bd screening via DNA probe* above).
  - Collect liver, kidney, and skin samples for *Ranavirus* testing (see *Ranavirus screening via DNA probe* above).
  - If organized by a veterinarian, additional samples can be submitted for electron microscopy (in glutaraldehyde fixative), viral culture (special media required), or micronutrient analysis (frozen organ tissue).
- *Carcass disposal:* For carcass disposal, institutions should follow appropriate necropsy procedures. Accepted tissue disposal options include formalin or alcohol fixation; incineration; alkaline tissue digestion; or disposal by a certified medical waste hauler.

**Treatments**

- *Bd* prophylaxis and treatment
  - *Prophylactic treatment*: This is suggested primarily 1) for amphibians that are coming from a known *Bd* positive collection or field site, 2) when PCR testing is not available, or 3) if animals positive for *Bd* are identified through testing. Specimens destined for release from PI to the wild require a minimum 10-day course of *Bd* treatment (listed below) to be completed immediately prior to release. Animals that test positive for *Bd* (and all their exposed cage-mates) should be treated and re-tested two weeks post-treatment. It is suggested that two to three PCR swabs be collected and submitted over the two week period to detect any low level residual infection. Multiple treatment cycles may be required to completely eliminate *Bd* infection.
  - *Method:* It is recommended to use the commercially available oral itraconazole formulations 10 mg/ml (1%) (Sporanox® oral solution; Itrafungol® oral solution) for dilution into Amphibian Ringer's Solution (ARS) (see Wright and Whitaker, 2001 for recipe). The commercial formulations are diluted to a 0.01% concentration with the bath applied for five minutes daily for 10 consecutive days (Nichols and Lamirande, 2000). For treatment, animals are placed into a plastic container and allowed to soak with their digits and ventral surface of their abdomen covered with the solution. After each treatment, animals are returned to cleaned and disinfected enclosures to prevent re-infection with *Bd* spores. Itraconazole treatment is not well-tolerated by some amphibian lifestages and species. In particular, treatment-associated deaths have been observed in tadpoles and sometimes recently metamorphosed animals. Use of lower itraconazole concentrations such as 0.005% have recently been shown to be effective for post-metamorphic amphibians (Pessier and Mendelson, 2010; Jones et al., 2012).
- Bacterial therapeutics
  Administer antibiotics with Gram-negative bactericidal activity prior to periods of stress; dosages and routes may vary based on species (Wright and Whitaker, 2001).
- Other pathogens or diseases
  Consult with staff veterinarian for specific treatments. Recently, it has become more apparent that many amphibian colonies are suffering from low level morbidity and mortality associated with nutritional issues such hypovitaminosis A and possible abnormal calcium/phosphorus metabolism. Necropsy and diet information are crucial to detecting and effectively correcting these more occult medical disorders.

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*Chapter 5*

# Creating Isolation Spaces for Amphibian Programs

Diane Barber

*AZA ATAG Chair, Curator of Ectotherms*
*Fort Worth Zoo*
*1989 Colonial Parkway*
*Fort Worth, TX 76110*
*dbarber@fortworthzoo.org*

## INTRODUCTION

Establishments have been keeping amphibians in captivity for more than a century for research and exhibit purposes. Over this time period, understanding of amphibians and their husbandry requirements has increased substantially, as has the focus on amphibian conservation. In 1984, the first Association of Zoo and Aquarium's (AZA) Species Survival Program® (SSP) was formed for the Puerto Rican crested toad (*Peltophryne lemur*) and in 1993 collaborative breeding efforts began for the Wyoming toad (*Anaxyrus (=Bufo) baxteri*). Both of these long-running reintroduction programs have served as models for intensive captive management of amphibians, and contributed to the premise of modern assurance colonies within AZA facilities.

The scope of amphibian assurance colony programs quickly expanded from regional to international following the International Union for the Conservation of Nature's (IUCN) *Amphibian Conservation Summit* (ACS) in 2005. This gathering was held in response to global amphibian declines that were documented through the 1980's and 1990's and the overall lack of action being taken to stop this rapid loss. The *Amphibian Conservation Action Plan* (ACAP) (Gascon et al., 2007) produced during that summit provided direction for the global community to address the amphibian extinction crisis. Over 500 threatened species were identified as candidates for immediate *ex situ* conservation action, and the zoological community was asked to begin creating biologically secure isolation spaces for assurance populations of these species. In 2006, the IUCN Conservation Breeding Specialist Group (CBSG) and the World Association of Zoos and Aquariums (WAZA) held the Ex Situ *Conservation Planning Workshop* in El Valle, Panama. From this workshop, guidelines were developed for biosecure containment of amphibian assurance colonies (Zippel et al., 2006). These guidelines, along with the first edition of the *Amphibian Husbandry Resource Manual* (Poole and Grow, 2008) and the *Manual for Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs* (Pessier and Mendelson, 2010) have laid the foundation for responsible management of amphibian assurance colonies, and are informing the way species are managed for reintroduction.

Throughout this initial push to take action, many organizations became confused about how to create appropriate amphibian isolation spaces and discouraged by the lofty goals that were identified in these various documents. A misunderstanding and an overwhelming sense

of burden has developed among some within the zoological community due to the large number of species in need and the resources perceived necessary to meet the suggested level of research and biosecurity for recovery efforts. While some have found it difficult to locate existing space for amphibians within their facilities, many more have found it challenging to identify the resources and support needed to build new structures. Despite this, many AZA-accredited institutions have found the means to respond to the call of action.

This chapter includes numerous examples of isolation areas that have been created for amphibians (descriptions and photos for each example are located at the end of this chapter). The individuals that have contributed to this chapter have encountered challenges that may be unique to their own situations, but have also found solutions through creative ingenuity that may be applicable to others. It is hoped that these examples, ranging from modified existing space to the creation of new facilities, can be used as tools for creating more places for amphibians world-wide.

## BIOSECURITY (BIOSAFETY) – WHAT DOES IT MEAN?

While the term "biosecurity" may conjure up images of people in hazmat suits breathing through respirators in sterile white rooms, it actually refers to "biosafety," which is defined as *safety from exposure to infection agents*. Although intimidating to some, everyone should remain diligent about following protocols to reduce the spread of infectious agents.

It is now widely understood that costly, sterile environments for amphibians are not necessary, however questions still arise regarding the appropriate level of biosafety. The answer is that it depends upon the situation. It is recommended that assurance or reintroduction populations remain in permanent isolation (i.e., species separated into a room within a building with species from other regions, or housed in an entirely separate building). When working with local or regional species, biosafety measures may be less extensive than with species from outside the region. Important considerations for designing amphibian isolation spaces include acceptable levels of biosafety/quarantine needed, and disposal of waste material and wastewater (for more information, see Chapters 3 and 4).

## TYPES OF FACILITIES

When starting a new program that requires dedicated amphibian space, review what areas and resources may already be available at the facility; these may range from new construction to modification of existing spaces. For some species, it may be preferable to create outdoor housing options, either in place of or in addition to, indoor housing to meet their needs. Most often these spaces can be found on the grounds of our existing facilities, but these same principles may be applied internationally, as needed. The following sections offer current examples of different amphibian isolation facilities, with the goal of serving as models and inspiration for new programs that may be developed.

### Outdoor Space

Perhaps the simplest and least costly type of facility is one that is created for a local species that can be housed outdoors. Working with local species that are exposed to the same local environments and pathogens are the most ideal, as biosafety measures are minimal and species can be housed outdoors within secure enclosures or areas. The Riverbanks Zoo (Example 1) is working with local dwarf sirens (*Psuedobranchus sp.)* which are native to their area and are housed in large, plastic stock tanks outdoors, and the Saint Louis Zoo (Example 2) in Missouri has created a large, fast-running stream on zoo property for a population of Ozark hellbenders (*Cryptobranchus alleganiensis alleganiensis*). These hellbenders are used as a study population to learn more about their natural history and potentially to augment local populations. Both of these semi-natural enclosures require little maintenance and provide moderate protection from predators. They also expose the animals to the same elements and light cycles they would experience in their natural habitat, which helps maintain animal health

and stimulate reproduction with little manipulation by caregivers. This type of situation is a LOW biosecurity risk (as described in Chapter 4), although dedicated equipment should still be used and proper hygiene techniques followed (see Chapter 3).

**Detached Spaces**

When referring to detached spaces in this document, the author is implying construction of a new or modified facility that is detached from existing buildings and may include shipping containers and sheds. These may be created in range country or on zoological grounds, and are an inexpensive alternative to construction/modification of an entire building. Careful planning is warranted, as hidden costs can often drive budgets higher than anticipated.

A small, prefabricated storage shed that can be located near a building with accessible power and water is easily assembled by novice staff and is less expensive than acquiring a refurbished and outfitted shipping container/freight trailer (i.e., pod). The Fort Worth Zoo created a shed for Puerto Rican crested toads (*Peltophryne lemur*) (Example 3), and the Toronto Zoo acquired a small prefabricated building and turned it into their *Amphibian Rescue Center* to expand the amount of isolation space available for species in need (Example 4).

Shipping containers can hold many amphibians, can be outfitted in one location and transported fully-assembled to another, and may be ready for use more quickly than a building that is newly constructed or modified. However, freight costs and the installation of power, water, and possibly sewer/septic system to a container can cause costly delays and logistical nightmares. To avoid headaches in the long-run, it is imperative to conduct thorough research (e.g., identify utility sources), determine the legal classifications (i.e., temporary or permanent) of the structures, and identify permitting requirements. Planning meetings with local officials prior to installation are important and may help resolve these issues. Memoranda of Understanding (MOU) or other contractual agreements should be considered when placing mobile units in remote regions to clearly define areas of responsibility for partners prior, during, and post- installation. Atlanta Botanical Garden purchased a fully-outfitted shipping container, the *FrogPOD,* which was placed on grounds for assurance colonies of frogs from Panama (Example 5) and Central Florida Zoo obtained an empty freight trailer which they dubbed the *Coqui Pad* and transformed it into an isolation space for endangered Eleuthrodactylid frogs from Puerto Rico (Example 6).

**Modified Spaces**

One of the easiest ways to create space for amphibian assurance populations with limited funds is to modify existing rooms or buildings because amphibians generally require less space compared to other vertebrates and most can be housed at ambient air temperatures. Vacant 8 x 8 ft office spaces or facilities built for other species could be transformed into amphibian holding areas. Although floor drains are nice, they are not required since many options are available for pumping or moving wastewater.

A number of facilities have repurposed spaces to increase their amphibian conservation capacity. Omaha's Henry Doorly Zoo transformed empty, drain-less hallways into twelve *Isolated Amphibian Rooms,* using greenhouse material for walls and plastic storage vats for water (Example 7). Northwest Trek Wildlife Park enclosed a free-standing garage to create a rearing room for local Oregon spotted frog (*Rana pretiosa*) tadpoles and an outdoor area for staging juvenile and adult frogs prior to release (Example 8). Jacksonville Zoo and Gardens modified a building that once held koalas into a *Save the Frogs* exhibit, featuring numerous interpretive graphics and behind-the-scenes viewing of isolation rooms (Example 9). Even historical buildings can be resourcefully altered at moderate cost while still maintaining their integrity; Toledo Zoo renovated the interior of a Depression-era museum into *Amazing*

*Amphibians,* which includes a large exhibit area for visitors and quarantined isolation space for four species assemblages (Example 10). Abandoned buildings *in situ* may be modified relatively quickly and at low cost if the overall structure is sound, providing an alternative to an outfitted shipping container; a vacated forest station in Madagascar provided the framework and foundation for a community-run amphibian rearing facility for local species (Example 11).

**New Spaces**

New construction dedicated exclusively to amphibians is rare. New amphibian spaces can be added to construction plans for an education building, animal hospital, primate facility, etc.; the options are limitless both in range and out of range country. Although exhibit space may help engage visitors, it is not crucial that amphibian assurance colonies are placed within public view.

The Detroit Zoo opened the first large-scale facility built entirely for amphibians in 2000, and the *National Amphibian Conservation Center* remains a popular exhibit for visitors today. The Atlanta Botanical Garden collaborated with the National Zoo in Chile to create breeding space for Darwin's frog (*Rhinoderma darwinii*) within a new building that also houses terrestrial invertebrates and flamingos (Example 12). The Fort Worth Zoo added four permanent isolation rooms for amphibians in an off-exhibit area within their newly constructed herpetarium, *Museum of Living Art* (Example 13), and the Phoenix Zoo created a conservation center for rearing and breeding rare Arizona species, which includes a large room for native amphibians (Example 14).

**Additional Resources**

Garnering support and obtaining resources for small creatures such as amphibians can be difficult, but it is not impossible. Amphibian programs need leaders to champion their species and conservation efforts within their own facility in order to garner resources comparable to those dedicated to other taxa. It is essential to share information and engage directors, boards, city officials, and/or state agencies regarding the need for action. Public outreach can expand a program's exposure, which may lead to unexpected external resources. Presentations and one-on-one conversations about amphibian declines have led to the development and completion of many of the amphibian facilities and programs presented in this manual.

Additional information on initiating amphibian conservation projects and identifying opportunities for grant support are available (Grow and Poole, 2008).

## CONCLUSION

A mass extinction event is occurring and space and resources for amphibians needs to be committed immediately. Although space and resources are at a premium, these obstacles can be overcome if allocated to prioritize amphibian conservation; the longer we procrastinate, or wait for others to take action, the more species will disappear. All that is needed to bolster conservation efforts for amphibians is foresight and creative planning with key personnel. Through examples and discussions, this chapter has provided a foundation for people to create new functional space for amphibians. Grab a hammer and start building today!

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**Example 1.**
**RIVERBANKS ZOO AND GARDEN NATIVE AMPHIBIAN HOLDING AREA**
Information and photos submitted by Scott Pfaff, Riverbanks Zoo and Garden

**Introduction**
Little is known about the status of dwarf sirens (*Pseudobranchus sp.*) in the wild and few are held in zoos. Although they have a wide range, habitat requirements may be narrow. In South Carolina, habitat preferences of *Pseudobranchus s. striatus* are limited to vernal ponds and pocosins occurring in pine flat woods and long-leaf pine forest. These habitats are currently undergoing significant alteration due to forestry practices and coastal development. *P. s. striatus* is listed by the South Carolina Department of Natural Resources as a state threatened species. By maintaining colonies in outdoor enclosures (Figures 1 and 2), the Zoo has learned more about their reproductive strategies and husbandry requirements, and is prepared to offer assistance should there be a need to bolster wild populations or to help other species of dwarf sirens in the future.

**Type of Construction and/or Modification**
Modification of an existing outdoor service area for Riverbanks' *Aquarium Reptile Complex*

**Estimated Total Square Footage**
Approximately 1,500 sq. ft.

**Initial Set-up Costs for Facility**
About $2,000

**Major Challenges**
Exclusion of native predators including mink, raccoons, and natracine snakes.

**Useful Additions and Features**
The facility has close proximity to a water system that provides water directly from the Saluda River. The Saluda is classified as a *State Scenic River* and is relatively-free from contaminants. Access to the river water system allows the facility to use open water systems in the outdoors amphibian enclosures. Native amphibians are exposed to natural changes in photoperiod and temperature, and feed on the many invertebrates that colonize the tanks.

**Areas for Improvement (i.e., planned differently or improved)**

There is a need to secure all of the outdoor amphibian enclosures within a screen barrier to exclude small predators, yet allow entry of insects, natural light, rain, etc.

**Figure 1.** Stock tank enclosure with wire mesh lid removed.

**Figure 2.** Rubbermaid tub with wire mesh lid removed.

**Example 2.**
**THE RON GOELLNER CENTER FOR HELLBENDER CONSERVATION AT THE SAINT LOUIS ZOO'S WILDCARE INSTITUTE**
Information and photos submitted by Mark Wanner, Saint Louis Zoo

**Introduction**
The Saint Louis Zoo's WildCare Institute, *Ron Goellner Center for Hellbender Conservation*, has recently completed two outdoor streams in partnership with the Missouri Department of Conservation, U.S. Fish and Wildlife Service, and private donors. Each stream is run on a unique aquatic system and will house two different river populations of Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) (Figure 1). Hopefully, this new addition to the Zoo's hellbender facility will build upon earlier successes with head starting, husbandry, and propagation programs.

**Type of Construction and/or Modification**
New construction completed in September 2011

**Estimated Total Square Footage**
Each stream is about 40 ft. in length. A new 400 sq. ft. building was constructed to house the life support equipment.

**Initial Set-up Costs for Facility**
Approximate costs for the construction of streams, life support building (Figure 2), and life support equipment were $200,000.

**Major Challenges**
Since the stream construction was just finished, no hellbenders had been introduced at the time of this writing.

**Useful Additions and Features**
The hellbenders will be exposed to natural seasonal changes in photoperiod and temperature, as compared to those housed in the indoor facilities. The streams are deep enough for staff to snorkel to monitor the hellbenders, and are outfitted with chillers, boilers, UV sterilizers, carbon towers, bag filters, and outdoor bio-towers. Wastewater is treated using chlorine

infusion. Stainless steel hydraulic lids were added after the initial construction was completed, are lifted manually, and will contain hellbenders while excluding predators (Figure 3).

**Figure 1.** Hellbender ready for transport

**Figure 2.** Interior view of life support building

**Figure 3.** Hydraulic lids

**Example 3.**
**FORT WORTH ZOO TOAD SHED**
Information and photos submitted by Diane Barber, Fort Worth Zoo

**Introduction**
This outdoor tool shed is a relatively inexpensive way to isolate a group of amphibians and can be placed next to a building for easy access to electricity and water (Figure 1). Similar units can be purchased at any major hardware store or on-line, and have the flexibility to be shipped or transported as an unassembled kit if needed in range-country.

**Type of Construction and/or Modification**
This kit was purchased in 2004 from a local hardware store, and was constructed on site by staff. Built to house Pecos pupfish, it is now used for a group of Puerto Rican crested toads (*Peltophyne lemur*). The shed's interior surfaces were easily disinfected using a large steam cleaner prior to moving in the toads. Custom wooden shelves were constructed along the walls to accommodate glass aquariums and lighting. The shed is equipped with a sink and water filtration/storage area (Figure 2), and is cooled/heated by small portable units.

**Estimated Total Square Footage**
This is a single room that is about 96 sq. ft. (Figure 3)

**Initial Set-up Costs for Facility**
Estimates for a similar set-up today would be approximately $2,220, not including labor for construction, or electrical and plumbing installation. Expenses are estimated as follows:

| | |
|---|---|
| Metal Racks | $400 |
| Roof shingles | $150 |
| Flooring material | $200 |
| Lighting/timers | $400 |
| Tanks/lids | $280 |
| Sink and fixtures | $60 |
| Water storage tubs | $60 |
| Water filtration | $120 |
| Window air conditioner | $250 |
| Small Heater | $300 |

**Major Challenges**

The shed lacks a central floor drain, so tank drains are routed to the sink and the linoleum covered floor is mopped. If the air conditioner or heater malfunctions, room temperatures fluctuate quickly, and so staff must be diligent about monitoring the building.

**Useful Additions and Features**

These easy-to-assemble units are available with windows/skylights, which would provide natural light cycles.

**Areas for Improvement (i.e., planned differently or improved)**

As space for a growing population may be a limiting factor, the size of the unit selected should reflect program needs. A hoseable floor surface with a central drain would be an improvement for keeper staff. An added screened vestibule or covered entryway would also be beneficial.

**Figure 1.** Window air conditioner powered by nearby building.

**Figure 2.** Water filtration and storage area.

**Figure 3.** Interior view of shed.

**Example 4.**
**TORONTO ZOO AMPHIBIAN RESCUE CENTER (ARC)**
Information and photos submitted by Bob Johnson, Toronto Zoo


**Introduction**
This building was purchased to expand isolated space for amphibians at our facility.

**Type of Construction and/or Modification**
This is a prefabricated building that was purchased new (Figure 1).

**Estimated Total Square Footage**
The *Amphibian Rescue Center* has a total of 280 sq. ft., containing two isolation rooms (Figure 2) that are 90 sq. ft. each and a vestibule (Figure 3).

**Initial Set-up Costs for Facility**
The building costs were approximately $84,000 (USD), shelving and tanks were about $1,500, and roughly $3,000 was spent to run the water lines and power to the facility.

**Useful Additions and Features**
If needed, the HVAC system allows for manipulation of temperatures in order to hibernate amphibians.

**Areas for Improvement (i.e., planned differently or improved)**
Several issues arise when temperatures are lowered to hibernate adult amphibians. First, the room becomes too cold for the juvenile amphibians, which has been overcome by staging the hibernation in the following manner: the room temperature is lowered by 10-15 F, which is still comfortable for the juveniles; once stabilized, the adults are moved to separate aquatic chiller systems where they are further cooled, while the room is returned to a normal temperature range for the growing juveniles. Second, high humidity within the rooms at low temperatures is also problematic as moisture condenses on the walls.

As space has become a limiting factor, the rooms should have been made about three feet larger so that three more tanks or a water reservoir could have been added.

**Figure 1.** Moving the Amphibian Rescue Center onto the concrete pad

**Figure 2.** Interior amphibian room

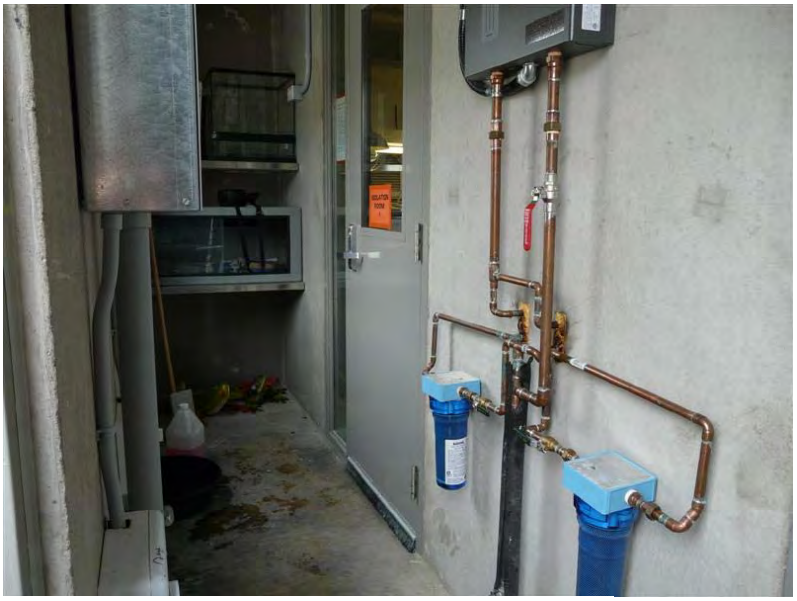

**Figure 3.** Interior vestibule

**Example 5.**
**ATLANTA BOTANICAL GARDEN'S FROGPOD**
Information and photos submitted by Robert L. Hill, Atlanta Botanical Garden


**Introduction**
The *FrogPOD* was purchased in order to house an assemblage of amphibians that were collected in Panama as an assurance population. This off-exhibit facility currently houses approximately 200 juvenile to adult animals, but could potentially house many more if needed.

**Type of Construction and/or Modification**
In 2008, the shipping container was purchased new as a complete, fully equipped/outfitted unit. Doors, windows, floor drain, and electrical outlets were installed by the company offering the unit, while plumbing, enclosures, and additional "after market" accessories were installed on-site.

**Estimated Total Square Footage**
Approximately 3,200 sq. ft.

**Number of Isolation Rooms**
The pod includes two rooms: an 800 sq. ft. entry room used for general storage and changing footwear, and a single 2,400 sq. ft. amphibian room (Figure 1).

**Initial Set-up Costs for Facility**
$53,000

**Major Challenges**
Inadequate heating and cooling systems were initially installed and the grated flooring/drain system has been problematic.

**Useful Additions and Features**
The heating and cooling problems have been remedied by upgrading to a more powerful split-unit heat pump, and installing back-up window air-conditioning units and small space heaters, to be utilized as necessary. The box shape of the unit makes design and layout of

enclosures quite simple, as the pod lacks the odd columns or strangely placed corners often found in many herp buildings.

**Areas for Improvement (i.e., planned differently or improved)**

Modification of the grated floor would be nice, as drainage has been a constant problem due to the lack of solid-surface floor material. The initial purchase of a split-unit heat pump would have solved many of the heating and cooling issues from the start. In areas that may reach warm seasonal temperatures for extended periods of time (i.e., the southeastern USA), inexpensive window a/c units may prove to be inadequate.

**Figure 1.** Amphibian holding room

**Example 6.**
**CENTRAL FLORIDA ZOO'S COQUI PAD**
Information and photos submitted by Jen Stabile, Albuquerque BioPark

**Introduction**
The Central Florida Zoo has partnered with the University of Puerto Rico - Rio Piedras to conserve several species of Eleuthrodactylid frogs that are on the verge of extinction. The *Coqui PAD* units were installed at the Central Florida Zoo to house assurance colonies of coqui as part of *The Coqui Initiative*.

**Type of Construction and/or Modification**
A refrigerated semi-truck trailer was purchased from a salvage company and the interior was remodeled in-house. Construction is in progress at the time of this writing (Figures 1 and 2).

**Estimated Total Square Footage**
384 sq. ft.

**Number of Isolation Rooms**
There are three rooms within the unit: the entrance room which serves as a keeper changing and storage area (64 sq. ft.); a quarantine room (64 sq. ft.); and the main frog room (240 sq. ft.)

**Initial Set-up Costs for Facility**
Initial costs for facility, prior to permitting inspections, were approximately $16,125. Actual costs for retrofitting the entire unit in order to comply with permit requirements (see Major Challenges) brought the final total to $29,770 (including labor). Initial costs are detailed below:

| | | |
|---|---|---|
| **Basic Structure** | | |
| Insulated trailer (48x8x8 ft.) | $2,200 | |
| Hurricane straps | $250 | |
| Foundation - cement/block | $1,500 | |
| Sealant for roof | $150 | |
| Wall construction | $200 | |
| PVC wall covering (48x8 ft.) 32 pcs | $3,376 | |
| Screws, rubber washers, and silicone seal | $200 | |
| Metal exterior doors w/deadbolt, 3 pcs | $600 | |
| **Electrical/Plumbing** | | |
| Power upgrade | $1,800 | |
| GFI breakers | $150 | |
| HVAC unit (512 cubic ft.) | $3,000 | donation |
| Window unit for QT | $400 | |
| Lighting – interior, exterior, and emergency | $350 | |
| Outlets, 25 total | $125 | |
| Instant hot water heater | $215 | |
| Filtration | $100 | |
| Misc. plumbing, check valves, and lighting | $500 | |
| Hose, 3 pcs | $60 | |
| Wet/Dry shop-vacuum | $82 | |
| Utility Sinks, 3 pcs | $180 | |
| **Furnishings** | | |
| Naturalistic Terrarium (12x12x12 in.), 10 pcs | $420 | |
| Naturalistic Terrarium (18x18x18 in.), 13 pcs | $1,010 | |
| Naturalistic Terrarium (18x18x24 in.), 5 pcs | $460 | |
| 18" hood lights (2) bulbs, 3 pcs | $420 | |
| Racks (36x14 in.), 6 pcs | $360 | |
| Racks (48x24 in.) ,6 pcs | $420 | |
| Cabinets, 4 pcs | $400 | |
| Desk | $100 | |
| Anti-fatigue rubber mats (36x36 in.), 32pc | $640 | |
| Footbath | $100 | |

**Major Challenges**

When the trailer unit was originally placed on site, the wheels were removed, placed on blocks, and hurricane-strapped to the concrete foundation pad. By doing this, a "permanent" structure was inadvertently created, which was then held to different permitting standards, including compliance with Americans with Disabilities Act (ADA) guidelines and required electrical upgrades. Since retrofitting the unit to be ADA compliant was not feasible, a decision was made to reattach wheels to the trailer, reclassifying the structure as "temporary" per the city's definition. These unexpected permit requirements created costly delays and expensive upgrades.

**Useful Additions and Features**

Although this is an off-exhibit facility, one side of the trailer was placed in line with the public fence with the intent to paint it with a giant mural describing *The Coqui Initiative* (Figure 3).

**Areas for Improvement (i.e., planned differently or improved)**

It would have been nice to have installed some skylights. It is also recommended to double check permitting regulations prior to building installation, even when dealing with structures intended to be "temporary."

**Figure 1.** Inside of trailer during construction

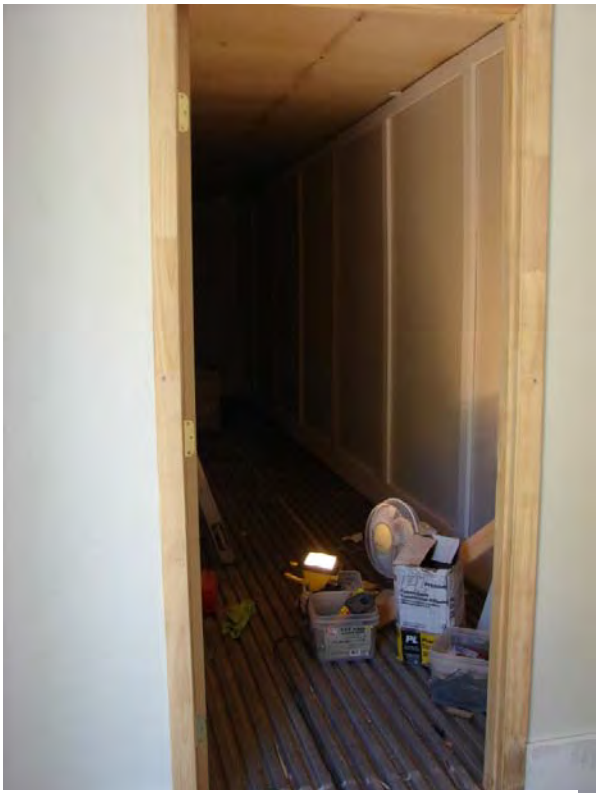

**Figure 2.** Inside of trailer during construction

**Figure 3.** Side of trailer from public side, to be painted with a mural

**Example 7.**
**OMAHA'S HENRY DOORLY ZOO'S ISOLATED AMPHIBIAN ROOMS**
Information and photos submitted by Jessi Krebs, Omaha's Henry Doorly Zoo

**Introduction**
Following the 2006 *Amphibian* Ex Situ *Conservation Planning Workshop* in El Valle, Panama, Omaha's Henry Doorly Zoo responded to the call-to-action and immediately established dedicated amphibian rooms within existing buildings on zoo grounds. The *Isolated Amphibian Rooms* (IARs) have become a working model for the application of the recommended standards in a zoo or aquarium setting. Each of the IARs holds one species or an assemblage of species from the same geographical area.

**Type of Construction and/or Modification**
IARs are versatile rooms constructed out of commercially available greenhouse materials with all construction completed by zookeepers. They have been set-up within the hallways of an unused, existing building on the Henry Doorly Zoo grounds.

**Estimated Total Square Footage**
Total square footage is over 4,200 sq. ft.

**Number of Isolation Rooms**
Currently there are 12 rooms that vary in size from 44 sq. ft. to 160 sq. ft. with the potential to add three more rooms.

**Initial Set-up Costs for Facility**
Current 2011 prices are approximately $8,000 to set up an 8x8 ft. unit. For estimating purposes, the rough expenses are as follows:

| | | |
|---|---|---|
| Room materials | $1,200 | |
| Shelving | $300 | |
| Heater/AC | $850 | |
| Frog tanks | $150 | each x 18 = $2,700 |
| Lighting | $220 | each x 9 = $1,980 |
| Plumbing | $500 | |
| Electrical/duct work | $250 | |
| TOTAL for one room | $7,780 | |

**Major Challenges**

Biggest challenge has been to staff the area and get outside help to complete projects.

**Useful Additions and Features**

IARs at the zoo range from 8x4x8 ft. (2.4x1.5x2.4 m) in size to 10x16x8 ft. (3x4.9x2.4 m). The walls are made of 1.5x1.5 in. (3.8x3.8 cm) hollow-aluminum tubing overlaid with two-ply Lexan® sheeting (Figure 1 and 2). Individual walls are joined together with 1 in. (2 cm) aluminum angle pieces (Figure 3). Commercially purchased storm doors are used to access each room. All joints and cracks are sealed with 100% silicone to prevent water from leaking into common areas or into other isolation rooms. Seals are pressure-tested before installation of equipment and animals and visual inspections are ongoing to maintain biosecure levels. The storm door is placed at the lowest point and the one-inch threshold allows each room to hold at least 175 gallons (796 L) before overflowing into a common hallway with a drain. List of items used for the construction of an 8x8 ft. room:

| | |
|---|---|
| Cap[1] | 18 @ 8 ft. (2.4 m) |
| Splice[2] | 3 @ 8 ft. (2.4 m) |
| Lexan®[2] | 6 @ 6x8 ft. sheets (1.8x2.4 m) |
| Aluminum Tubing[2] | 18 @ 8 ft. [1.5x1.5 in. (3.8x3.8 cm); 1/8 in. (0.3 cm) thick] |
| Storm door | |
| Hardware, screws, washers | |

Portable heating/air condition units are used to control the ambient temperature in each room (Figure 4). Units can be purchased with different BTU ratings for different size rooms: 8x8x8 ft. (2.4x2.4x2.4 m) rooms use 10,000 BTU units; the 10x16x8 ft. (3x4.9x2.4 m) use 12,000 BTU units[3]. Also visible in Figure 4 is the designated footwear for within this room. Footwear that is easy to disinfect is changed as the keeper crosses the room threshold. Once the shelving[4] is installed, tubs and lids[5] used for amphibian enclosures are fabricated from food-grade polycarbonate material to prevent the leaching of toxins sometimes found in plastic materials (Figure 5). Though glass fish-tanks may be a less expensive, the polycarbonate tubs are far more durable and versatile, making them suitable for housing terrestrial or aquatic species. Drilling each tub does not require a specialized drill bit nor do they crack or break as easily as glass. The volume of the tanks used ranges from 5-16 gallons.

The drain for each enclosure runs into a common piping system located under every shelf. Drain system lines are 2 in. (3 cm) diameter to allow for large volumes of water to pass through them without backing up into adjacent enclosures (Figure 6). The drain systems pipes all run into the wastewater collection tub (Figure 7).

[1] www.stuppy.com
[2] www.statesteel.com/omaha.htm
[3] www.sunpentown.com
[4] www.samsclub.com or from materials acquired at local hardware stores
[5] www.rubbermaidcommercial.com/rcp/products/detail.jsp?rcpNum=3328

Lighting on every rack system is made available in two forms: compact florescent or MR16 track lights[6] above each shelf to provide ultraviolet light, and small heat lamps on each enclosure to offer basking sites for species requiring higher temperatures (Figure 8).

**Areas for Improvement (i.e., planned differently or improved)**

At the time of design of the IARs, wastewater treatment was highly recommended for all amphibian isolation facilities, however current practices only require wastewater treatment for those facilities which do not directly flow into a municipal sewage system. The information on the IAR wastewater treatment plan that follows is offered for facilities desiring such an example.

A sink combination is used to collect all wastewater from each isolation room and is created by stacking two inexpensive utility sinks together (Figure 7). The bottom tub (without legs) is set directly on the floor un-drilled. The second sink (with legs) is set within the tub below, and plumbed to drain into the lower tub without splashing. A sump pump[7] with an automatic on/off switch is set within the lower tub to pump wastewater to the *Central Treatment Station* (Figure 9). The upper tub can be plumbed for use as a working sink if desired, or else dedicated hose-lines can be run into each room and provide filtered source-water.

All water is treated coming into and out of the IAR facility at the *Central Treatment Station*. A large water container is used to hold reconstituted reverse-osmosis (RO) water that can be pumped to each room as needed (right side of Figure 9; See Chapter 1 for additional information on reconstituted RO water). Two barrels are used to collect all wastewater (center of Figure 9), which is then treated with household bleach for 12 hours before being released into the city sewer system (see Chapters 3 and 4 for more information on wastewater treatment). List of basic items used for influent and effluent water treatment within the room shown in Figure 9:

| | |
|---|---|
| RO water storage vessel | 300 gallons (1135 L) |
| RO filter system | |
| RO reconstitution feeder | |
| Wastewater treatment barrel | 2 @ 55 gallon (208 L) |
| Bleach feeder system | |

[6] 50 watt MR-16 style Eiko® bulb
[7] www.flotecpump.com

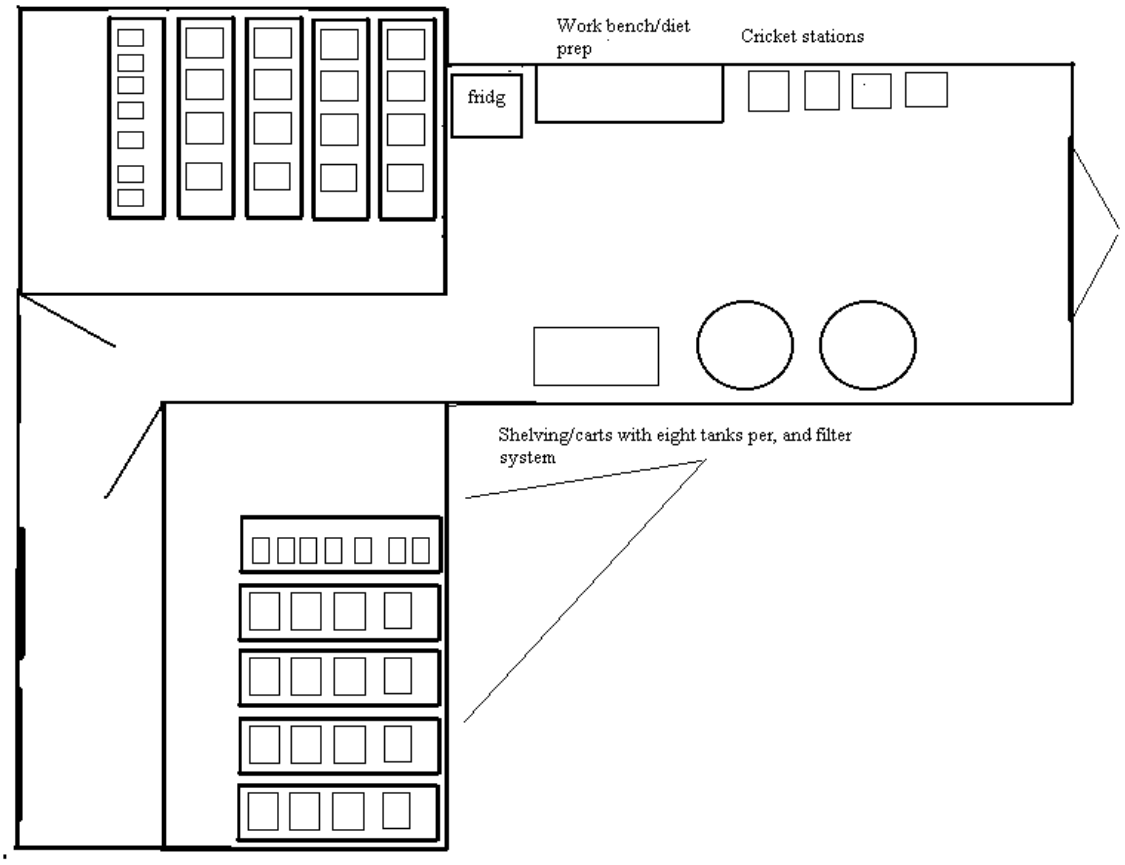


**Figure 1.** Floor plan of one Isolation Amphibian Room (IAR) with dedicated food preparation area.

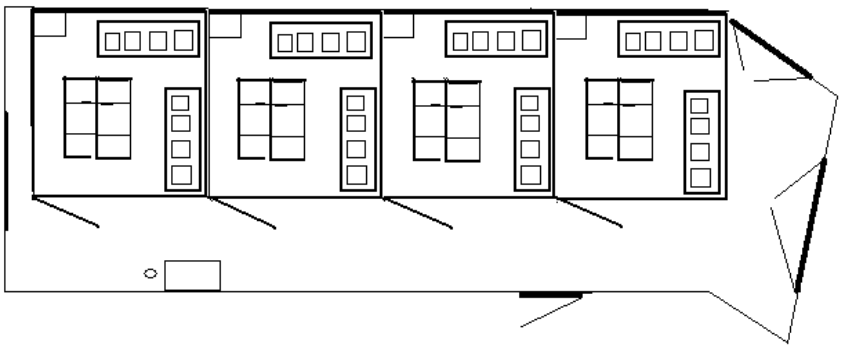

**Figure 2.** Floor plan of additional IARs.

**Figure 3.** Close-up of the 1 in. (2 cm) aluminum angle pieces holding the 1.5x1.5 in. (3.8x3.8 cm) aluminum tubing and storm door.

**Figure 4.** The portable heating/air condition unit and dedicated footwear placed in each room.

**Figure 5.** Shelving with amphibian enclosures

**Figure 6.** The over-sized drain system under each shelf being installed in the IAR.

**Figure 7.** IAR wastewater collection tub with sump pump below

**Figure 8.** Lighting accommodated into the racks


**Figure 9.** The building's water storage and *Central Treatment Station*

**Example 8.**
**NORTHWEST TREK WILDLIFE PARK'S OREGON SPOTTED FROG REARING FACILITY**
Information and photos submitted by Allison Abrahamse, Northwest Trek Wildlife Park

**Introduction**
The Northwest Trek Wildlife Park participates in the recovery efforts for the Oregon spotted frog (*Rana pretiosa*) with several other zoos and agencies. Since this is a regional species, they can be housed in outdoor enclosures. Although not every facility maintains Oregon spotted frogs in the same type of setting, this is one example of how facilities can accommodate local species.

**Type of Construction and/or Modification**
NTWP remodeled a free-standing garage into a workspace complete with sink, refrigerator, stove, and space necessary for diet preparation and storage of materials. The insulated garage is temperature controlled and utilizes a specialized filter to eliminate excess iron from local well water. In addition, a chain link kennel was constructed outside of the garage to house adult frogs (Figure 1). The frogs are reared in 300 gallon holding tanks[8] within the kennel, with each tank able to hold approximately 100 animals. Separate 300 gallon reservoir tanks with de-chlorinated water are adjacent to each respective holding tank, along with a dedicated pump used for water changes (Figure 2). The size of kennel needed is relative to the number of tanks used for rearing. To prevent predation from above, the kennel is covered with nylon netting, and hot wire runs around the top edge to deter climbing predators (e.g., raccoons).

**Estimated Total Square Footage**
A total of 1,400 sq. ft. is used for the NTWP Oregon spotted frogs. The converted garage is approximately 600 sq. ft., the enclosed kennel is approximately 400 sq. ft., and the space with the reservoir tanks is about 400 sq. ft.

[8] Rubbermaid® stock tanks

**Initial Set-Up Costs for Facility**

The total cost to create this facility with four holding tanks and four reservoir tanks was approximately $7,745, which does not include costs for the "kitchen/storage" area remodel, labor, or installation. Cost approximations are detailed as follows:

| | |
|---|---|
| Fencing | $1200 |
| Netting | $150 |
| Hot wire | $150 |
| 300 gallon stock tanks | $375 per tank |
| 800W titanium aquarium heaters | $45 per tank |
| Temperature controller | $28 |
| Timers | $25 |
| Thermometers | $20 |
| Air pump | $210 |
| Air stones/tubing | $35 per tank |
| Water pump | $200 each |
| Hoses | $20 |
| Lids (tight construction mesh w/bungee ring suspended over PVP pipe diameter support rod) | $100 each |
| Cleaning equipment (buckets, nets, turkey basters, etc.) | $30 per tank |
| Net-pens (pool noodles and aquarium nets; houses approx. 20 eggs each) | $12 each |
| Water quality testing kits | $50 each |

**Major Challenges**

One challenge faced was a lack of amphibian expertise on staff, leading to a significant amount of time invested in reading related literature and talking to more experienced colleagues. Other beginners should not be surprised to do the same, and it is recommended to have one or more mentors from outside the institution if in-house experience is lacking. Even with more experience under one's belt, collaboration with other facilities is still one of the best resources.

The next challenge faced was an unexpected amendment to the rearing protocols that now incorporated the use of supplemental heat to accelerate the development of tadpoles and frogs. Adding heat precipitated the need to start improvising and acquiring items that had not been budgeted. Staff time dedicated to addressing this issue immediately ballooned, and the ultimate demands for electricity required significant investment in electrical infrastructure among other features.

Another challenge faced was related to water quality. The facility is located in a rural area and uses well water which requires extensive water quality testing; this would normally be conducted by a municipal water department in suburban or urban areas. Because the well water has high iron content and is not processed by a municipal water treatment plant, an iron filter was installed to produce an acceptable influent to the Oregon spotted frog facility.

**Useful Additions and Features**

The literature was mixed with respect to the need for full-spectrum lighting however, NWTP was conservative and invested in full-spectrum lighting for rearing eggs and young tadpoles indoors (Figure 3). Floating net-pens (Figure 4) in outside holding tanks negate the need for artificial full-spectrum lighting.

A good quality water test kit with freshly stocked reagents is recommended.

In the author's opinion, institutional commitment for all departments and at all levels is the single most important *feature* needed to succeed.

**Areas for Improvement (i.e., planned differently or improved)**

Have seasoned amphibian experts on staff prior to beginning a project, ideally with experience working with the same or a closely related, species. Shy of that, employ committed seasoned generalists, who are willing to learn, hustle, adapt, communicate, and persevere.

**Figure 1.** Outdoor Kennel that houses adult frogs.

**Figure 2.** Each holding tank for live individuals (inside the fencing) has its own separate reservoir tank (outside the fencing) that holds de-chlorinated filtered water.

**Figure 3.** Tadpole rearing enclosures inside of remodeled garage.

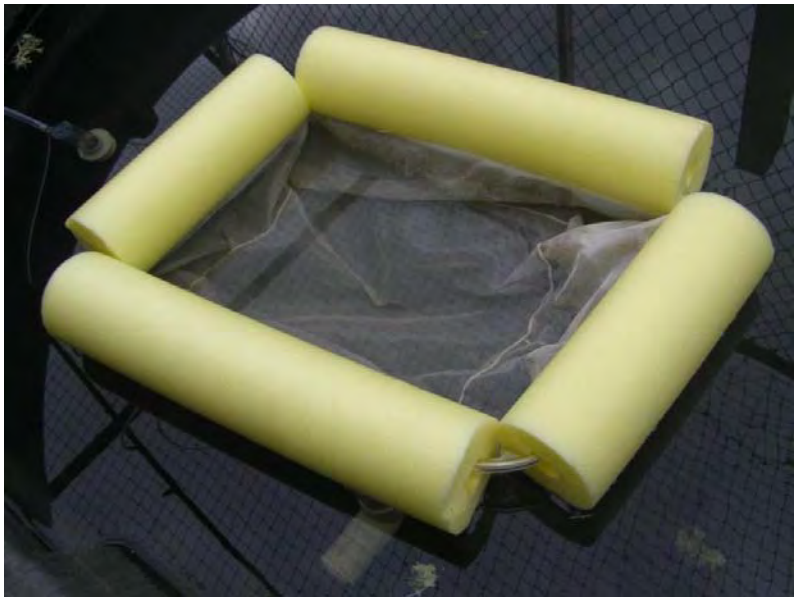

**Figure 4.** Net-pens currently used for tadpole rearing outdoors.

**Example 9.**
**JACKSONVILLE ZOO'S SAVE THE FROGS EXHIBIT**
Information and photos submitted by Dino Ferri, Jacksonville Zoo and Gardens


**Introduction**
The Jacksonville Zoo decided to retrofit an old koala exhibit and dedicate the space to amphibian conservation.

**Type of Construction and/or Modification**
Retrofit that was completed in 2008 for *Year of the Frog*, which included biosecure rooms with dedicated water treatment systems (Figure 1).

**Estimated Total Square Footage**
About 1,500 sq. ft.

**Number of Isolation Rooms**
Currently there are two isolation rooms (120 sq. ft. each; Figure 2), one exhibit room (80 sq. ft.; Figure 3), and a potential future fourth space (100 sq. ft.) that is currently used by the bird department as an egg incubation room.

**Initial Set-up Costs for Facility**
$30,000 to retrofit the entire building and set up the amphibian areas: $20,000 for building modifications and $10,000 for educational graphics, including a $2,500 mural (Figure 4).

**Major Challenges**
Securing the species for display in time for exhibit grand opening.

**Useful Additions and Features**
The public is able to view the amphibians in quarantine while in a comfortably air-conditioned building. The isolation rooms have skylights, which not only help illuminate the space, but provide more natural photoperiods for the amphibians.

**Areas for Improvement (i.e., planned differently or improved)**
More space would be nice in order to work with additional species.

**Figure 1.** Sink and water treatment area for each room

**Figure 2.** Isolation room


**Figure 3.** Exhibit room

**Figure 4.** Graphic wall

**Example 10.**
**TOLEDO ZOO'S AMAZING AMPHIBIANS**
Information and photos submitted by R. Andrew Odum, Toledo Zoo


## Introduction

In May 2008, the Toledo Zoo opened its permanent dedicated *Amazing Amphibians* (AA) facility in the Depression-era Museum of Science building constructed in 1937. The area of AA is roughly divided into thirds, with one-third each dedicated to biosecure amphibian populations, keeper service, and public education. The first goal of this facility is to provide a conservation education experience for the Zoo visitor focused on both local amphibian species and the worldwide diversity of the Amphibia. The second goal is to provide four isolated and discrete biosecure amphibian facilities to serve as areas for *ex situ* conservation efforts. Three of these isolation areas are currently dedicated to ongoing reintroduction programs.

## Type of Construction and/or Modification

New space within an existing building.

## Estimated Total Square Footage

Total square footage of AA is 2,000 sq. ft.

## Number of Isolation Rooms

There are four biosecure isolation rooms that vary in size from 100-170 sq. ft.

## Initial Set-up Costs for Facility

The total project budget was approximately $750,000. This amount included the structure; caging; display; interpretive graphics; environmental control systems; plumbing; new electrical service and electrical installations; keeper service areas; and biosecure facilities.

## Major Challenges

Available funds did not cover the labor for equipping and fitting the reserve areas, life support systems, and biosecure facilities; these were completed by keeper staff. Fabrication of the elevated floors and curbs in AA required a more expensive alternative as the existing floors of the old Museum building were inadequate support the weight of concrete.

**Useful Additions and Features**

AA features four separate isolation rooms connected to a common pre-isolation hallway (Figure 1). The hallway has a unique access point from the public area with no direct connection to other animal areas. This hallway is used for staff preparation prior to entering isolation areas and doubles as a dedicated invertebrate culture area and storage. It includes a curtained area for privacy, where keepers wash and change into scrubs prior to entering any isolation rooms.

Elevated floors with curbs in all service areas allow for drains and prevent water on the floor from flowing between service areas. A structure of wood framing, plywood, and composite-layered epoxy-flooring forms service area floors with drain troughs, and continues up each wall for approximately one foot, creating a waterproof barrier. Each isolation room entrance has an 8 in. sill to prevent water from entering or exiting the room at the doorway (Figure 2). The walls were made of 6 in. metal framing, 5/8 in. plywood sheathing, and covered with a fiberglass reinforced panel (FRP) system with molding. This creates a waterproof seal which facilitates cleaning. All walls were insulated with fiberglass, and a clear plastic vapor barrier was installed inside the plywood on both sides.

Each independent isolation room has its own air handling system, using commercially-available refrigeration equipment. There is no fresh air provided in the design of the isolation rooms. Instead, air exchanges are provided by opening the door during servicing to exchange air with the hallway. A constant unidirectional flow exhausts air that is exchanged between the hallway and isolation rooms, preventing cross-contamination between rooms as only one isolation room door is opened at a time. Direct-wired time-clocks and thermostats provide a day/night temperature change.

Hot and cold domestic water supplies, carbon-filtered water (for dechlorination)[9], and reverse osmotic (RO) water are available in AA. All wastewater from enclosures, keeper areas, and biosecure rooms is routed to a single common drain system in the floor trough, allowing disinfection if necessary; however, currently the wastewater is routed to the municipal water treatment plant and no further treatment is considered warranted (Pessier and Mendelson, 2010).

A new electrical service was installed for the entire facility. All room circuits were protected with ground fault circuit interrupt breakers (GFCI) installed within the panels. The electrical outlets are installed in the raceway system at a height of about six feet and separate circuits were provided for timers and continuous power.

Shelves were created out of composite decking 2x4 in. lumber and stainless steel fasteners. The shelf tops are made of half-inch high-density polypropylene purchased from a local plastic suppler. Cage lighting was provided by track light systems and 50W Eiko® EXT/SU 12V halogen bulbs with the lens removed (Browne et al., 2007).

All the glass tanks[10] were drilled and fitted with ¾ in. PVC bulkhead fittings. A false floor was installed and the drains were piped to external standpipes to prevent cage flooding (Figure 3). A quick-drain method was provided by the PVC ball valves installed below the level of the tank bottom. These drains are piped into a common drain with an air-gap to prevent siphoning of water between cages. The common drain empties into a floor trough drain piped

[9] 3M backwashing filter from Grainger® - Part # 3P971
[10] Zoo Med® terraria typical

to the building's wastewater system. Rain systems were later installed using the carbon filter water supply.

Staff maintain biosecure isolation for each of the rooms by donning dedicated footwear, head coverings, and clean scrubs; disinfecting hands before and after animal exposure; and utilizing latex gloves between each cage (Figure 4).

**Areas for Improvement (i.e., planned differently or improved)**

One issue that was not anticipated was the amount of dew produced each morning when the temperatures changed from nighttime lows to daytime highs; addressed by using off-the-shelf domestic dehumidifiers to help dry the air during temperature transitions.

**References**

Browne, R., R. Odum, T. Herman, and K. Zippel. 2007. Facility Design and Associated Services for the Study of Amphibians. ILAR journal 48(3): 188.

Pessier, A. and J. Mendelson, Eds. 2010. A Manual for Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs. Apple Valley, MN, IUCN/SSC Conservation Breeding Specialist Group.

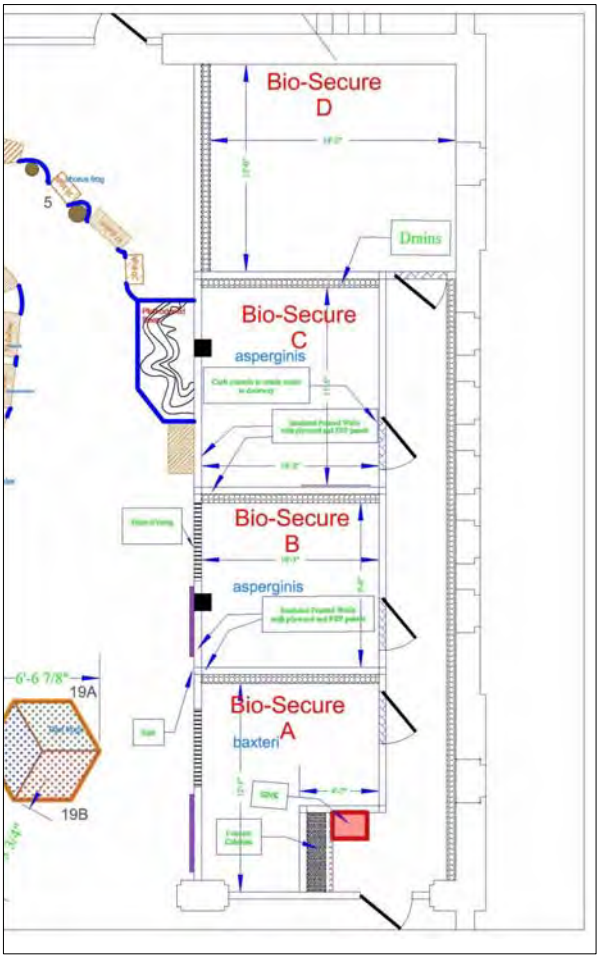


**Figure 1.** The isolation area hallway is used to access the four individual biosecure rooms in AA.

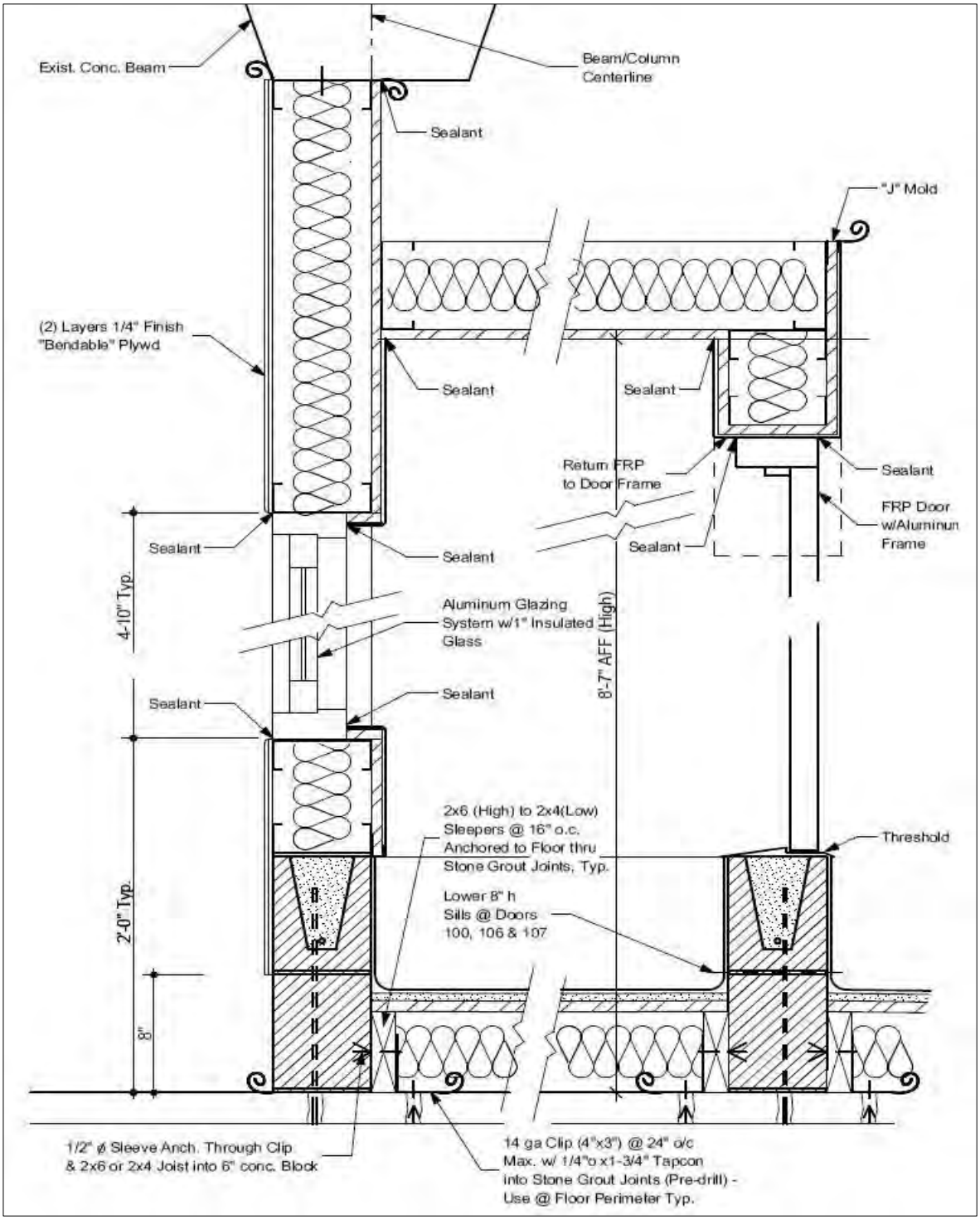


**Figure 2.** Detail for the floor, wall, and door structures. Note the elevated door threshold to prevent water from exiting and entering the room under the entrance door.

**Figure 3.** Each tank has its own standpipe overflow created out of "T" and flexible PVC, serviced by a quick-drain valve. All these drains flow into a two-inch common drain pipe created by drilling a hole in the pipe and loosely fitting the ¾-inch drain into the pipe. This prevents siphoning between cages.

**Figure 4.** Elevated door threshold into an isolation room from common hallway. Note the dedicated footwear for hallway and each room.

**Example 11.**
**MADAGASCAR AMPHIBIAN CONSERVATION PROJECT**
Information submitted by Jennifer Pramuk, Woodland Park Zoo; Photos by Devin Edmonds

**Introduction**
Slightly more than one quarter of Madagascar's amphibian species are threatened with extinction. Habitat destruction and over-harvesting are the greatest factors contributing to this dramatic decline. The impending threat of amphibian chytrid fungus (*Batrachochytrium dendrobatidis; Bd*), which is responsible for many of the world's recent amphibian population declines and extinctions, is also of great concern. With the generous financial assistance of multiple organizations, the community-run Malagasy organization Association Mitsinjo has constructed a captive breeding facility in the Mitsinjo-managed Analamazaotra Forest, two km outside of Andasibe village in east-central Madagascar. This is the first in-country captive breeding and amphibian conservation project of its kind in Madagascar, and the facility will house captive populations of threatened amphibians to safeguard against current threats as well as the future introduction of *Bd*. The goal is for the facility to become a center for training and education in a bio-region of Madagascar which contains tremendous amphibian diversity and endemism. This project also has begun to develop additional value-added components, including collaborative efforts with the Amphibian Specialist Group (ASG) to perform local and country-wide *Bd* testing as a first line of detection for the disease in Madagascar. So far, four frog species have been selected for this project through discussions with colleagues that developed *A Conservation Strategy for the Amphibians of Madagascar* (ACSAM) and the ASG of Madagascar: *Blommersia blommersae*, *Boophis pyrrhus*, *Heterixalus betsileo,* and *Mantidactylus betsileanus*. These species are absent from zoological collections and little or nothing is known of their husbandry. They were selected based on their varied life histories and presumed correlated differences in captive care parameters to provide technicians with a diverse set of husbandry experience during training. Technicians from Andasibe have been trained in proper quarantine, biosecurity, and acclimation protocols, in addition to animal health procedures that ensure the health of each frog before it is transferred to permanent housing. The project's second phase will involve development of educational materials and graphics to accompany an exhibit that will display some of the frogs to the public. Tourists will be charged a nominal fee to view the exhibit and these admission funds will augment the livelihoods of Malagasy technicians running the facility.

**Type of Construction and/or Modification**
This project was almost completely new construction, but was built upon the foundation of an old, abandoned forest station in the Analamazaotra Forest Reserve. The foundation included a partial concrete pad and walls. The facility is solidly constructed of bricks, mortar, wood, plaster, concrete, and a zinc sheet metal roof. Basic construction of the facility was completed in late winter of 2011.

**Estimated Total Square Footage**
The total square footage of the facility is 185 sq. m (Figure 1).

**Number of Isolation Rooms**
The facility includes three primary biosecure rooms for culturing live foods (Figure 2), maintaining captive populations of frogs (Figure 3), conducting husbandry research, and for quarantine (Figure 4).

**Initial Set-up Costs for Facility**
Initial set up costs were approximately $45,000 which included facility construction, on-going maintenance, tanks, shelving, electrical wiring, amphibian husbandry materials, and equipment for the production of live food. Early and significant funding was received from the AZA Conservation Endowment Fund and more recent support was awarded by Conservation International, Wildlife Conservation Society, Woodland Park Zoo, Cleveland Metroparks Zoo Africa Seed Grant, Durrell Ivoloina Training Course Small Grant, Tree Walkers International Amphibian Conservation Partnership, and the Amphibian Ark Seed Grant.

**Major Challenges**
Construction of municipal water and electrical lines to the facility has been the greatest challenge to the project. Politics have delayed the delivery and installation of a water line in the facility for almost seven months. Electricity is an even greater financial obstacle as the electric company wants to charge a substantial amount for installing power lines to the facility. Alternative energy, such as from solar panels, are being investigated to power the facility. Fortunately, a recent grant from Conservation International will allow the purchase and installation of large rain barrels that will be used for the primary water supply.

Additional challenges include sourcing materials in-country (e.g., plastic boxes, aluminum framing, glass, and silicone). Materials such as prefabricated aquariums, which are taken for granted in the United States, are nearly impossible to obtain in Madagascar.

**Useful Additions and Features**
A rainwater collection system is being developed so that the technicians do not have to hand-carry water to the facility. Solar panels are being sought to use as a reliable power source.

It was necessary to construct an exclusion fence for zebu and other large animals to prevent damage to the facility itself.

**Areas for Improvement (i.e., planned differently or improved)**
Improved communication with other international organizations prior to project initiation would have been tremendously beneficial. Relationships that were damaged due to poor communications early on are now being repaired; this could have been prevented if plans were more thoroughly vetted with other stakeholders from the start.

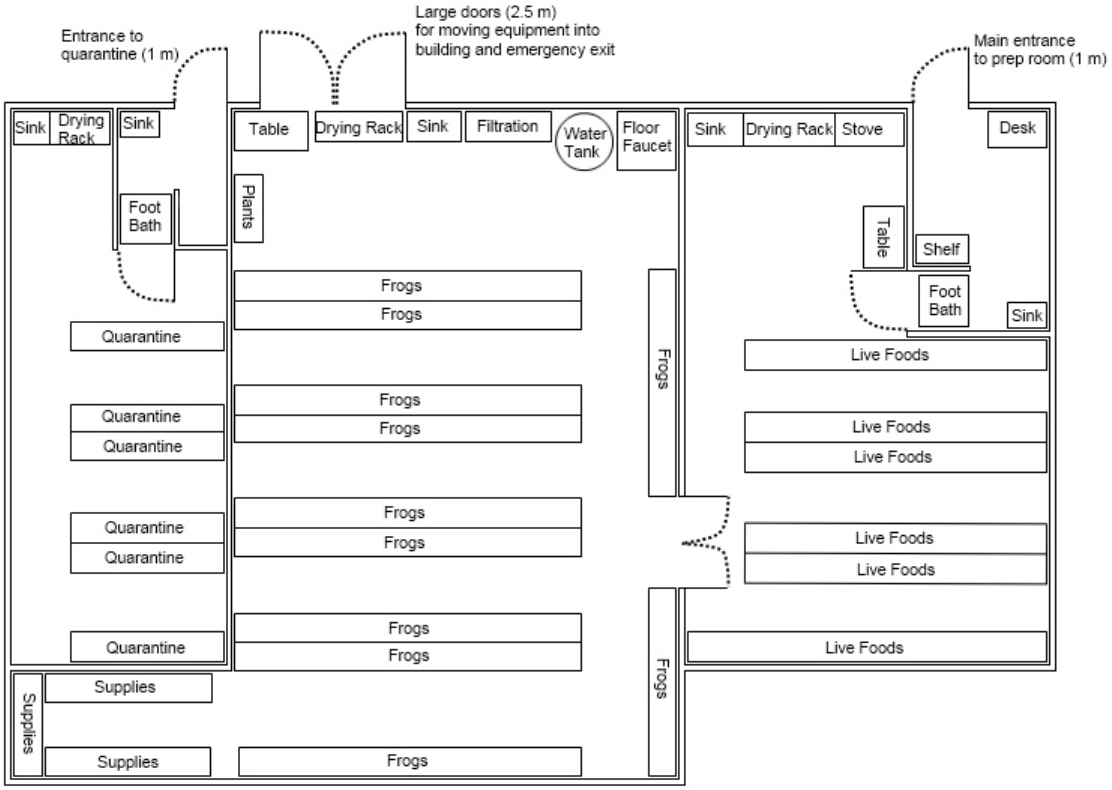


**Figure 1.** Floor plan

**Figure 2.** Insect Room

**Figure 3.** Anteroom to quarantine

**Figure 4.** Quarantine Room

**Example 12.**
**DARWIN'S FROG BREEDING FACILITY AT THE NATIONAL ZOO OF CHILE**
Information and photos submitted by Danté Fenolio, Atlanta Botanical Garden


**Introduction**
The Atlanta Botanical Garden helped the National Zoo of Chile (Santiago Zoo) complete a breeding facility for Darwin's frogs (*Rhinoderma darwinii*). This facility also includes two other areas dedicated to rearing Chilean flamingoes and feeder insects.

**Type of Construction and/or Modification**
A newly constructed building completed in 2009

**Estimated Total Square Footage**
The amphibian level is approximately 14 sq. m.

**Number of Isolation Rooms**
Two amphibian rooms, approximately 7 sq. m each. There is an additional insect culture room on the first level of the building that is also about 7 sq. m.

**Initial Set-up Costs for Facility**
The actual construction cost for all three levels of the building was approximately $360,000 (USD). The cost to equip (e.g., shelving, lighting, aquariums, etc.) the inside of the amphibian isolation rooms cost approximately $15,000 (Figure 1).

**Major Challenges**
One of the major challenges with setting up this facility was the development of preventative measures in the event of an earthquake. This resulted in the installation of a self-starting generator (Figure 2), a gravity-fed water storage system (Figure 3), and securing aquarium racks to walls. These additions were proven effective after a large earthquake impacted the area shortly after building construction, and left the zoo without electricity and water for a short period of time.

The amphibian isolation rooms are visible to the public through a large glass window (Figure 4). Initially, the window received a lot of sunlight in the mornings and overheated the rooms. Therefore, a large 4x1 m banner was installed above the upper portion of the windows, providing adequate shade from the sun and also serving as a graphic panel that describes the Darwin's frog project to visitors in both English and Spanish.

**Useful Additions and Features**

A large statue of a Darwin's frog was placed outside of the amphibian exhibit to attract children to the area and entice parents to read and interpret the graphics to them (Figure 5).

**Areas for Improvement (i.e., planned differently or improved)**

More space for juvenile frogs will probably be needed in the future. Two additional units are planned to accommodate at least six other critically endangered Chilean amphibians.

**Figure 1.** Lab service area

**Figure 2.** Generator

**Figure 3.** Water storage tank

**Figure 4.** Exhibit view

**Figure 5.** Frog sculpture

**Example 13.**
**FORT WORTH ZOO AMPHIBIAN QUARANTINE ROOMS**
Information and photos submitted by Diane Barber, Fort Worth Zoo

**Introduction**
The Fort Worth Zoo's herpetarium features four amphibian isolation rooms. Each room houses a different species that is part of a reintroduction program (Figure 1), or serves as an assurance population with the potential for reintroduction in the future. A limited number of staff has access to these rooms, and biosecurity procedures are followed when servicing each area (Figure 2). This type of single-room approach can be applied to retrofit spaces within existing buildings (e.g., hospitals, bird or mammal areas, office spaces, etc.) at minimal costs.

**Type of Construction and/or Modification**
The amphibian quarantine rooms are within the newly constructed *Museum of Living Art* (MOLA), which opened in 2010.

**Estimated Total Square Footage**
760 sq. ft.

**Number of Isolation Rooms**
Three rooms are approximately 10x12 ft., and the forth room is 10x20 ft. The amphibian room corridor is about 203 sq. ft., and the remaining 560 sq. ft. is dedicated quarantine space (Figure 3) divided into four rooms.

**Initial Set-up Costs for Facility**
The rooms were part of a multi-million dollar construction project. Costs for equipment to initially outfit all four rooms [reverse-osmosis (RO) unit, water storage, sink, racks, tanks, lights, etc.] were approximately $8,225.

**Major Challenges**
Originally poured concrete curbs were requested to prevent water moving from the corridor under the doors, but due to Americans with Disabilities Act (ADA) regulations, it was not possible to add that feature. Instead, floor sweeps were added to the bottom of all doors and

thresholds were installed on the interior floors to prevent water transfer. Daily cleaning and quarantine procedures overcome any minor water breeches that may occur.

**Useful Additions and Features**

Ultra-violet (UV) penetrating skylights were installed in each of the rooms. Each room has its own sink, hose, water filtration/storage area, and floor drain (Figure 4). A multitude of timed and untimed outlets are on each of the walls. Although the amphibian room's air conditioning system is shared with the adjacent kitchen area, the ducts that supply air to each of the rooms is equipped with a High-Efficiency Particulate Air (HEPA) filter.

**Areas for Improvement (i.e., planned differently or improved)**

It would have been ideal to have separate heating and cooling systems for each of the rooms, but it was too costly; instead, the four rooms are on a single thermostat. Bigger spaces, or additional rooms, are preferred where possible.


**Figure 1.** Rooms are permanently marked to identify occupants and remind staff to follow protocols.

**Figure 2.** Dedicated footwear, gloves, and lab coat are worn by staff.

**Figure 3.** This large quarantine room with skylight houses larval hellbenders on the shelves, and includes a 12 ft. long enclosure to accommodate adults.

**Figure 4.** Service area within each quarantine room includes a cabinet, sink, hose, and water filtration/storage vat.

**Example 14.**
**THE ARTHUR L. AND ELAINE V. JOHNSON FOUNDATION CONSERVATION CENTER AT THE PHOENIX ZOO**
Information and photos submitted by Tara Sprankle, Phoenix Zoo

**Introduction**
This facility was built for head starting and captive breeding/rearing of Arizona native species.

**Type of Construction and/or Modification**
This conservation center is a new facility completed in the summer of 2007.

**Estimated Total Square Footage**
The building is 3,000 sq. ft. There are four separate labs used for Arizona species, one of which is a 1,250 sq. ft. room dedicated to amphibians (Figure 1).

**Initial Set-up Costs for Facility**
The total cost for construction of the building was approximately $750,000. The initial cost to outfit the amphibian room was about $5,150, detailed below:

| | |
|---|---|
| Shelving | 1,400 |
| Rubbermaid food storage tubs | 1,000 |
| Filters | 750 |
| Misc. supply | 2,000 |

**Major Challenges**
The facility has insufficient storage space.

**Useful Additions and Features**
The amphibian room is equipped with a deep sink (bathtub), which is ideal for disinfecting tanks and cleaning filters. The lab is visible to visitors so they can see the work being done with the amphibians, including rearing tadpoles (Figure 2). Solar panels on roof help power the facility (Figure 3).

**Areas for Improvement (i.e., planned differently or improved)**
More storage space and additional electrical outlets would be useful.

**Figure 1.** Interior view of the amphibian lab

**Figure 2.** Tadpole rearing tanks

**Figure 3.** Exterior of the facility exhibiting solar panels

*Chapter 6*

# Amphibian Population Management Guidelines

Created at an amphibian population management workshop
10-11 December 2007
San Diego Zoo, San Diego, California, U.S.A
Sponsored by the Amphibian Ark

These guidelines are subject to further refinement.
Please check with the Amphibian Ark (www.amphibianark.org) for any updates.

**Representatives from:**
Amphibian Ark (AArk)
Association of Zoos and Aquariums (AZA)
Australasian Regional Association of Zoological Parks and Aquaria (ARAZPA)
AZA Population Management Center, Lincoln Park Zoo
Chicago Zoological Society - Brookfield Zoo
Conservation Breeding Specialists Group (CBSG) of the IUCN Species Survival Commission
Fresno Chaffee Zoo
Zoological Society of London (ZSL), London Zoo
Minnesota Zoological Garden
Sandfire Dragon Ranch
Toledo Zoological Gardens
World Association of Zoos and Aquariums (WAZA)
Zoological Society of San Diego

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# TABLE OF CONTENTS



## Meeting Attendees & Other Important Contacts

| Attendee | Job Title | Institution | E-mail | Area of Expertise |
|---|---|---|---|---|
| Kristin Leus | CBSG Europe Programme Officer; Population Management Advisor; Co-Chair Amphibian Population Management Committee | Copenhagen Zoo; European Association of Zoos and Aquaria (EAZA); Amphibian Ark (AARK) | Kristin@cbsgeurope.eu | **Primary Contact for Amphibian Population Management;** Population Biology |
| Kristine Schad | Population Biologist; Co-Chair Amphibian Population Management Committee | AZA Population Management Center; Amphibian Ark (AARK) | kschad@lpzoo.org | **Primary Contact for Amphibian Population Management;** Population Biology |
| Bob Maillouix | Owner | Sandfire Dragon Ranch, California | | Amphibian Husbandry |
| R. Andrew Odum | Curator | Toledo Zoological Society | RAOdum@aol.com | Amphibian Husbandry |
| Mike Ready | | Sandfire Dragon Ranch, California | | Amphibian Husbandry |
| Andy Snider | Director of Animal Care and Conservation | Fresno Chaffee Zoo | asnider@fresnochaffeezoo.com | Amphibian Husbandry |
| Louise Bier | Consulting Population Biologist | AZA Population Management Center | lbier@lpzoo.org | Population Biology |
| Kevin Johnson | Amphibian Ark Taxon Officer | Australasian Regional Association of Zoological Parks and Aquaria (ARAZPA) & Amphibian Ark (AARK) | kevinj@amphibianark.org | Software Development |
| Richard Gibson | Curator Lower Vertebrates and Invertebrates; Amphibian Ark Taxon Officer | Zoological Society of London (ZSL)* & Amphibian Ark (AARK) *recently moved to Chester Zoo | Richard@amphibianark.org | Conservation Biology, Species Prioritization |
| Bob Lacy | Conservation Biologist | Chicago Zoological Society & IUCN/SSC Conservation Breeding Specialist Group (CBSG) | rlacy@ix.netcom.com | Population Biology; Management Software Development |
| Sarah Long | Senior Population Biologist | AZA Population Management Center | slong@lpzoo.org | Population Biology |
| Brandie Smith | Senior Biologist | Association of Zoos and Aquariums (AZA) *recently moved to National Zoological Park | smithbr@si.edu | Group Management; Population Biology |
| Bob Wiese | Director of Collections | Zoological Society of San Diego | BWiese@sandiegozoo.org | Population Biology |
| Kevin Willis | Biological Programs Director | Minnesota Zoo | Kevin.Willis@state.mn.us | Population Biology |
| Sharon Baker | Curatorial Assistant | Zoological Society of San Diego | SKBaker@sandiegozoo.org | |
| Becky Bryning | Senior Systems Analyst/Programmer | Zoological Society of San Diego | RBryning@sandiegozoo.org | |

# INTRODUCTION

Compiled by Brandie Smith, Association of Zoos and Aquariums

The maintenance of genetic variation within a population increases the probability of both its long- and short-term survival and that of the comprising individuals. As the basis for evolution, genetic variation allows populations to adapt to changing environments (Allendorf 1986; Lewontin 1974; Selander 1983) and many studies have shown its benefits to individual fitness (Hedrick et al. 1986; Allendorf and Leary 1986; Ralls et al. 1995; Lacy et al. 1993; Wildt et al. 1987). Small populations are especially susceptible to loss of genetic variation through the process of genetic drift (Nei et al. 1975). This random fluctuation in allele frequencies can greatly impact the genetic composition of small populations, hastening their demise.

The science of population management has been greatly advanced through programs developed for captive populations (Ballou and Lacy 1995; Lacy et al. 1995; Ballou and Foose 1996). Professionally managed zoos and aquariums maintain populations of animals for display, conservation, research, and education purposes (Hutchins & Conway 1995). Because these populations are small and widely dispersed, they are managed cooperatively through captive breeding programs such as the Association of Zoos and Aquariums (AZA) Species Survival Plan (SSP®) and Population Management Plan (PMP), the Australasian Regional Association of Zoological Parks and Aquaria (ARAZPA) Australasian Species Management Program (ASMP), and the European Association of Zoos and Aquaria (EAZA) Endangered Species Programme (EEP). Through these programs, specific breeding recommendations are made to help maintain sustainable populations that are genetically diverse and demographically stable.

The goal of captive genetic management is to stop evolution. More specifically, management is intended to minimize changes in a population's gene pool to retain as many of the genetic characteristics of the population's original founders as is possible (Ballou and Lacy 1995). Founders are individuals that are assumed to be unrelated and that have living descendents. It is currently feasible to slow the loss of genetic diversity in pedigreed populations through intense management. The genetic constitution of the entire population can be examined from information found in the pedigree, animal-by-animal breeding recommendations can be made, and the effects of long-term management evaluated.

The current strategy used worldwide by cooperative captive breeding programs to minimize loss of genetic diversity pairs individuals according to a mean kinship (MK) value (Ballou and Lacy 1995). Under this strategy, an individual's genetic importance can be assessed based on the number and degree of relatives that the individual has in the population. Individuals with the lowest mean kinship are priority breeders. Mean kinship has proven to be the best strategy at maintaining genetic diversity in pedigreed populations, tested against alternatives in both a computer simulation (Ballou and Lacy 1995) and on living organisms (Montgomery et al. 1997). Mean kinship is only effective when the entire pedigree is known and pairings can be controlled. This strategy is practical for many species in captivity including elephants, komodo dragons, and vultures, but impractical for species with insufficient information or those where we have less control of pairings. For these species, recommendations are more lenient by attempting to minimize inbreeding and prevent fixation of alleles in subpopulations.

The class Amphibia includes three orders – anurans (frogs and toads), caecilians, and caudates (salamanders and newts) – and covers over 6000 species which exhibit a wide range of natural histories and reproductive strategies. Although some amphibians follow a reproductive model that allows individual identification, known parentage, and controlled pairings, many more do not. In addition, behavioral considerations are very important in maintaining captive amphibian populations and specific environmental cues may be needed to achieve reproduction in captive breeding programs (Pramuk and Gagliardo 2008). Consequently many species of amphibians in captivity do not fit the mean kinship model and a diverse range of specific management techniques must be implemented to maximize the maintenance of genetic diversity. These techniques are the topic of these 'Amphibian Population Management Guidelines.'

****Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

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## DATA MANAGEMENT FOR AMPHIBIAN POPULATIONS

Compiled by Sarah Long and Kristine Schad, AZA Population Management Center

Management of amphibian populations in zoos depends on databases called "studbooks." A studbook is a record of the chronological history of a single managed species. It is compiled from institutional data based on all known information about each individual in the population, including its relationships to other individuals and dates of birth and death. Studbooks provide the data for demographic and genetic analyses, which in turn help ensure a population's survival in zoos & aquariums. Studbooks tracking individuals can be created easily using SPARKS or PopLink software (see below).

### DEMOGRAPHY

**Definition:** Demography is the science of how a population's size, structure, and distribution have changed in the past and how they might be expected to change in the future.
**Goals:** To achieve and maintain desired population sizes, stable age structures, and biologically appropriate sex distributions.
**Implementation:** Determining and recommending the number of births or hatches that will help the population achieve its demographic goals.
**Information required:** In order to plan the appropriate number of births or hatches, we need to know information about the animals' reproductive capabilities (e.g., age at first and last reproduction, litter/clutch size, interbirth interval, probabilities of breeding at various ages, etc.) and mortality rates (probability of dying at different ages, lifespan, etc.).
**Raw data required:** Reproductive and mortality data are essentially derived from four critical pieces of information—birth dates, parentage or other monitoring of reproductive performance, death dates, and sex.

### POPULATION GENETICS

**Definition:** Population genetics is the study of how a population's genetic structure, more specifically, how the frequencies of alleles (variants of genes), are distributed within and between populations, as well as how these distributions change over time.
**Goal:** To preserve gene diversity and avoid inbreeding.
**Implementation:** Gene diversity is maintained and inbreeding is avoided through the careful selection of breeding individuals or groups.
**Information Required:** In order to determine which males, females, or groups should be reproducing, we need to know their pedigrees. Pedigrees give us information about the comparative genetic value of each animal or group (how unique or common their alleles might be) and their relatedness to each other. If individual or group parentage is unknown or uncertain, then the other variables become important as clues for how animals may be related (e.g., source, location, and date of birth or immigration, etc.).
**Raw data required:** Pedigree and relatedness information is based on parentage data that are traced through the generations from the living animals back to the wild born/hatched founders.

### RECORD KEEPING FOR INDIVIDUAL MANAGEMENT

- Individually identify, mark, and record the different founders and their descendants, if possible.
    - Use any feasible method – transponders, implanted tags, photographs of unique markings, separate enclosures (both for individuals and groups), etc.
- Parentage of individuals
    - Record sire and dam whenever possible
- Sex of each individual, if possible
- Birth/hatch date, Location, and Origin
    - If wild caught, record date, site location, possible relationship to other wild caught individuals (i.e., several amphibians captured from same water source), and date animal entered captivity
    - If zoo/aquarium born, record parents and their wild caught locations
- Locations and transfers of individuals (i.e., moving to a new enclosure, mixing with a new individual/group, transferring to a new institution)
- Enclosure composition (i.e., who housed with whom, in breeding situation or not)

- Death date, Location, and Cause
    - Note if death was due to various natural causes vs. managed cull

**RECORD KEEPING FOR GROUP MANAGEMENT**

INITIAL DATA ENTRY

- Identify, mark, and record the different founders or founder groups with unique identifiers.
    - Use any feasible method –label separate enclosures, etc.
- Track enclosures, locations
- Origin or parentage of group (founders from the wild, split from another group, combinations of other groups). Be as specific as possible to track group pedigree and genetic composition.
    - If wild caught, record date, site location, possible relationship to other wild caught individuals (i.e., several amphibians captured from same water source), and date animals entered captivity.
- Group composition (who is housed with whom)
- Generation number (e.g., founder, F1, F2, etc.)

ONGOING DATA COLLECTION

- Take regular census counts (weekly, monthly, or as often as is feasible) and record dates associated with these counts to identify:
    - Number in each life stage (i.e. eggs/clutches, metamorphs, adults)
    - Number of each sex (if possible)
    - Number of deaths
        - Cause of death (various natural causes vs. managed cull)
- Record any events and dates associated with them
    - Transfer of groups (new enclosure, location information)
    - Splitting groups (record ID & location of new subgroups)
    - Merges of groups (record ID & location of new combined group)
    - Reproductive or developmental events

Ballou J.D. and Foose T.J. 1996. Demographic and genetic management of captive populations. In Kleiman D.G., Lumpkin S., Allen M., Harris H., Thompson K. (eds.) *Wild Mammals in Captivity*. Chicago, IL: University of Chicago Press. p. 263-283.

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**SOFTWARE**

**SPARKS** (Single Population Analysis and Record Keeping Software, ISIS) is a DOS-based computer program designed to be used in the management and analysis of studbook databases. A studbook is an electronic record of the history of a captive population. It includes information on every individual in a population, including pedigrees and dates of birth, death and transfers between institutions. The studbook traces the entire history of each individual in a population; these collective histories describe the population's genetic and demographic identity.
SPARKS software is available to all members of ISIS (International Species Information System) on the ISIS software installation CD.

**PopLink** is a Windows-based computer program designed to be used in the management and analysis of studbook databases. Similarly to SPARKS, PopLink can help maintain, analyze and export the data for a captive population that are relevant to its genetic and demographic management. PopLink can import and export a studbook from/to SPARKS, the current software used to manage studbook datasets. Studbook keepers can use PopLink to track and maintain all the data relevant to an individual species within zoos. Population biologists can use PopLink to store analytical data, the version of the studbook used in the genetic and demographic analyses that management decisions are

based on. PopLink includes many tools that assist with the development and maintenance of the analytical data necessary for management. PopLink was developed by Lincoln Park Zoo.
PopLink is shareware that is distributed free of charge by Lincoln Park Zoo from www.lpzoo.org/poplink. Any questions or comments can be directed to software@lpzoo.org.

**PM2000** software provides a suite of tools for genetic and demographic analysis and management of pedigreed animal populations (a studbook). PM2000 combines the capability of the MS-DOS programs GENES (written by Robert Lacy, Chicago Zoological Society), DEMOG (written by Laurie Bingaman-Lackey and Jon Ballou, National Zoological Park), and CAPACITY (written by Jon Ballou), as well as adding some new features. PM2000 was developed by JP Pollak (Cornell University), Bob Lacy, and Jon Ballou.
PM2000 is shareware that can be obtained from http://www.vortex9.org/pm2000install.zip

Other population management tools are currently being developed, by Zoological Society of London, Chicago Zoological Society, National Zoo (Washington) and likely elsewhere. It is expected that these additional tools will be available soon to help with management of amphibians, especially those species for which pedigrees cannot be accurately tracked and managed at an individual level

**GENETIC MANAGEMENT OF AMPHIBIAN POPULATIONS**

Compiled by Sarah Long, AZA Population Management Center

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Generally, high levels of gene diversity are associated with GREATER/HIGHER values of the following:

- Number of founders (founders = unrelated individuals who help establish a population) (See Appendix A, Figures 1, 2, & 3)
- Proportion of breeding individuals (# breeding individuals / total # individuals) (See Appendix A, Figures 4, 5a, & 5b)
- Population growth rate (See Appendix A, Figures 6 & 7)
- Population size (starting size and target size) (See Appendix A, Figure 4)
- Number of offspring that survive to reproduce

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BASIC GUIDELINES FOR GOOD GENETIC MANAGEMENT:

FOUNDERS

- Start the population with at least 20 founders, ideally with an equal sex ratio (i.e., 10.10). (Note: throughout this document, the notation x.y will be used to signify x number of males and y number of females.) (See Appendix A, Fig. 1, 2, & 3)
    - This means at least 20 individuals (or groups of individuals) that are unrelated and that will successfully reproduce. Realize that many more than this number may have to be captured to ensure that 20 actually survive and successfully reproduce.
    - Collection of founders should be targeted towards obtaining as many unique lineages as possible (e.g., collect from different locations and, if possible, different sites at each location to reduce the probability of collecting related animals).

BREEDING – HOW MANY?

- Produce an equal number of offspring from each founder to equalize family sizes within the space available for the taxon. (See Appendix A, Figures 4, 5a, & 5b)
    - Produce at least 5 offspring per founder.
    - Keep numbers of offspring equal across founders – based on the amount of space available, divide spaces for offspring equally for each founder.

POPULATION GROWTH – HOW QUICKLY SHOULD THE POPULATION GROW?

- Species with short generation times (reproductive lifespan < 5 years) will need to have as many individuals produce as many offspring as fast as possible. A larger target population size is also beneficial (both to avoid demographic crisis and better retain genetic diversity). (See Appendix A, Figures 6 & 7)

ENVIRONMENT

- Environmental conditions should encourage reproduction and minimize unintentional selection in the highly altered zoo environment. However, conditions should also not be so narrow and rigid as to encourage unintentional selection to specific captive conditions. Variation in the captive environment will help maintain genetic variation and allow for testing of possible improvements in husbandry.

BREEDING – WHICH INDIVIDUALS/GROUPS?

- Once founders have successfully reproduced, keep these same pairs/groups together; do not mix and match unnecessarily. If potential founder pairs fail to breed successfully, then try other pairings and any other available manipulations to try to propagate their genes.
- Prioritize breeding the parental generation before the offspring.
    - Parents are always more genetically valuable than their offspring.
    - However, attempt to breed the 2nd generation before the founders die to test husbandry methods.
    - Descendants can be bred with founders when there are no other options.

- Prioritize underrepresented lineages (those with fewer descendants) for breeding and pair animals with similar genetic value:
    - If lineages are unequal, breed the smaller, underrepresented family lines with other underrepresented family lines.
    - If space allows, breed overrepresented family lines with other overrepresented family lines.

**Note: if individuals can be marked and individual pedigrees tracked, then breeding those with the lowest mean kinship will achieve several of the above goals.

CULLING

- Cull surplus offspring from the population when necessary in order to equalize founder lineages & to stay within target population size.
    - "Culling" is used here to refer to any method of permanently removing individuals from the primary breeding population, such as:
        - Transfer to any nonbreeding population (for research, display, etc.)
        - Release back into native environment when appropriate
        - Euthanasia (when animals are euthanized, biomaterials should be preserved, for example by depositing in the frozen zoo at San Diego Zoo, California, U.S.A. or Frozen Ark, University of Nottingham, UK)
    - To avoid selection prejudices:
        - The number of individuals/groups to be culled should be based on equalizing family size.
        - The selection of which individuals/groups to be culled from a family lineage should be <u>randomly</u> chosen (e.g. do not target for culling only the fast-developing tadpoles, the slowest swimmers, the ugliest specimens, etc.).
        - If individuals and pedigrees are tracked, then culling those animals with the highest mean kinships will achieve the two goals above
        - If culling is necessary, it should be done at the earliest life stage possible without compromising the stability and survival of the population.
    - Culling should follow appropriate disposition policies (government, institution, association, etc.).

SPECIAL CONSIDERATIONS FOR POPULATIONS MANAGED AS GROUPS - Basically, the same rules as described above apply to groups, but some special considerations are worth mentioning:

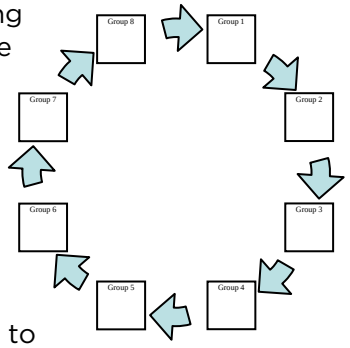
GROUP SIZE

- Keep group sizes as small as is effective for the biology of the species while meeting the husbandry needs for captive management
- Keep as many groups as space and reproductive biology allows.
- Equalize family size across groups by keeping clutch sizes as equal as possible.
- If successfully breeding individuals within groups can be identified, consider removing them from the group after they breed to allow other individuals to breed.

GROUP BREEDING STRATEGIES

There are several strategies to retain gene diversity in populations of group-living animals:

A. Once reproduction occurs, systematically transfer individuals among groups in a "round robin" manner (see figure). We recommend one of these methods:
    - Transfer about 5 individuals per generation – This number may need to be increased if mortality is high or fecundity is low.
    - Transfer all juveniles – Move all juveniles out of their natal group to establish new next-generation groups before they reach reproductive maturity.
    - Transfer all of one sex – Move all males (or females) from one group to the next group to avoid inbreeding with offspring and to mix genetic lines.



OR

B. Keep each unique founder group together indefinitely and allow them to interbreed (and inbreed) without mixing with other groups. This method does not imply maintaining the entire population of a species as a single group (i.e. do not put all your eggs in one basket). Rather, this method assumes some initial subdivision into many smaller groups, to safeguard against catastrophic events, and then moving forward with isolated group breeding. This strategy can maintain founder lineages in each group, but also involves the risks of rapid genetic loss, if some groups are lost, and quick, possibly deleterious, inbreeding. The population should be monitored for signs of inbreeding depression, so transfers to reverse inbreeding can be implemented if inbreeding is threatening success. Transfers may be necessary eventually if inbreeding depression develops.

OR

C. Split the starting founder population in half and follow both strategies A and B (above) to increase chances of breeding success.

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**DEMOGRAPHIC CONSIDERATIONS FOR MANAGEMENT OF AMPHIBIAN POPULATIONS**

Compiled by Lisa Faust, Alexander Center for Applied Population Biology, Lincoln Park Zoo

Establishing stable and viable populations of amphibians in captivity is initially dependent on working out the husbandry techniques to ensure survival and reproduction of wild-caught individuals. Once those techniques are established, the decision of how large a population should be maintained to ensure long-term viability should be considered, ideally for each individual population by an experienced population biologist. It is difficult to generalize about a single magic number that is a minimum viable population size from a demographic perspective because the demographic patterns of amphibian species in the wild fall into a wide range of life histories and the goals for a captive population may vary. Amphibian populations can be very "fast" species with high fecundity, high mortality, and large fluctuations in population size over time or "slow" species with lower fecundity and mortality which have more stable population sizes over time, or may lie somewhere in between the two life history extremes (for a good summary, see Green 2000).

However, demographic theory and some pre-existing research on amphibians can provide some minimal starting guidance on important demographic considerations for captive amphibian management. If a population's size is too small, it becomes susceptible to stochasticity, which is variability in survival and reproduction that can be due to demographic or environmental processes, which can result in further declines in population size or extinction. A general rule of thumb is that populations may be less susceptible to demographic stochasticity if they are at least 100 individuals (Morris and Doak 2002).

In addition, the total population size is not the only important determinant of a population's viability, as different life stages can be more or less important to long-term persistence. Biek et al. (2002) looked at several amphibian populations with a range in demography (although most would still be considered "fast" species) to determine the importance of different life stages to the population's long-term growth rate. They found that post-metamorphic vital rates were more critical to long-term growth than pre-metamorphic vital rates. This indicates that, once basic husbandry techniques have been worked out, improving survival rates in post-metamorphic stages is the most important objective for increasing the size of a captive population.

An additional important consideration for management of demographic risks includes protecting populations from catastrophic loss by essentially "not putting all your eggs in one basket". When setting up a new captive population, it would be extremely risky to setup the entire population in a single tank because of the risks from common catastrophes such as electrical failures, disease outbreaks, issues with water contamination, food problems, etc. These risks can be mitigated by spreading the population across multiple tanks in the same area, in multiple locations across an institution, or at multiple institutions.

Ultimately, once husbandry techniques have been perfected for new taxa, managers and population biologists should fully evaluate its population biology. A target population size can be set based on genetic goals, and then demographic management tactics can be planned to meet that target size goal based on the population's survival and fecundity rates. Amphibians can vary hugely with respect to fecundity, although husbandry considerations and high mortality of early life stages will narrow the range that reach metamorphosis. From a population management perspective, the important consideration for population growth is the number of progeny per brood that survive to breeding age. Culling of individuals from large broods to reduce numbers to a desired or manageable level should generally aim to leave equal numbers per brood, or should use mean kinship strategies to determine which ones to cull.

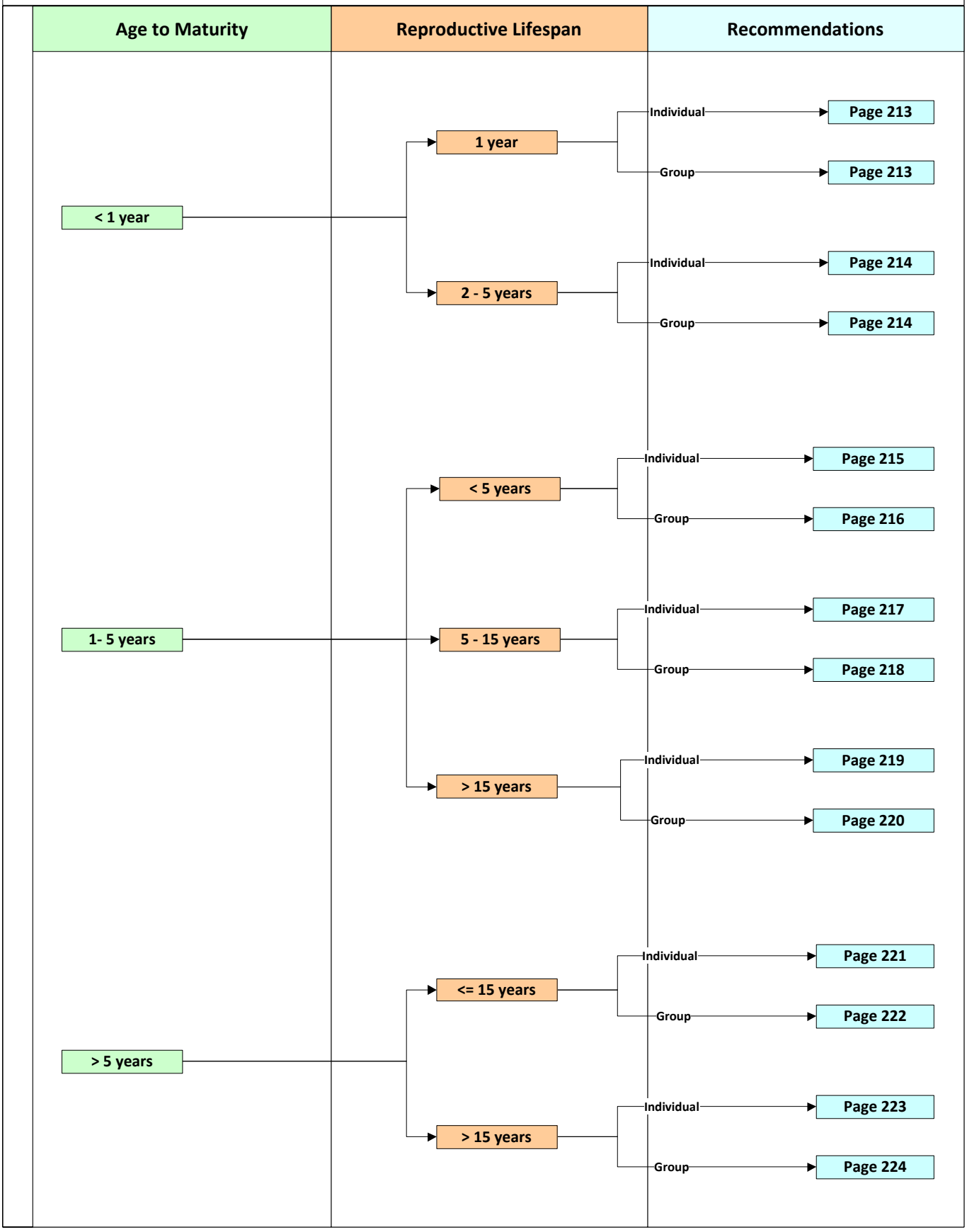
Biek R., Funk W.C., Maxell B.A., and Mills L.S. 2002. What is Missing in Amphibian Decline Research Insights from Ecological Sensitivity Analysis. Conservation Biology 16(3): 728-734.

Green D.M. 2000. "How do Amphibians Go Extinct" from L. M. Darling, editor. 2000. Proceedings of a Conference on the Biology and Management of Species and Habitats at Risk, Kamloops, B.C., 15 - 19

Feb., 1999. Volume One. B.C. Ministry of Environment, Lands and Parks, Victoria, B.C. and University College of the Cariboo, Kamloops, B.C. 490pp.

Morris W.F. and Doak D.F. 2002. Quantitative Conservation Biology. Sinauer Associates Inc. Sunderland, MA. 479 pp.

## Decision Tree




**Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| < 1 year | 1 year |

**Example Species:** ***Acris crepitans***

**Population Management Issue:** These species will lose genetic diversity very fast; so many founders and large population sizes will be needed.

## INDIVIDUAL MANAGEMENT

- Individual Management may not be feasible for these types of species. However, if you choose to manage individually use the group management recommendations below to ensure long-term viability of the population.

## GROUP MANAGEMENT

**How many founders to collect?**

- You want 50.50 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (i.e., if you expect 50% of the collected animals to survive and reproduce, you should collect 100.100 specimens.) Try to gather as even a sex ratio as possible.
- Keep founders in groups as small as possible (e.g., in pairs) to give equal breeding opportunity to all founders. If founders are kept in larger groups, you may need more founders to ensure 50.50 breeders.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 6 months and an effective population size of 0.15.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| 10 | 635 |
| 15 | 950 |
| 25 | 1590 |
| 40 | 2540 |
| 55 | 3490 |
| 70 | 4430 |
| 85 | 5480 |
| 100 | 6330 |

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size as quickly as possible due to short lifespan (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

Group Size

- Keep group sizes as small as is effective for the biology of the species-if possible try to maintain eight separate groups.
- Equalize family size across groups by keeping clutch sizes as equal as possible.
- If successfully breeding individuals within groups can be identified, consider removing them from the group to allow other individuals to breed.

Group Breeding Strategies: There are several strategies to retain gene diversity in populations of group-living animals:

A. Once reproduction occurs, systematically transfer individuals among groups in a “round robin” manner. We recommend one or more of these methods:

- Transfer about 5 individuals per generation – This number may need to be increased if mortality is high or fecundity is low.
- Transfer all juveniles – Move all juveniles out of their natal group to establish new next-generation groups before they reach reproductive maturity.
- Transfer all of one sex – Move all males (or females) from one group to the next group to avoid inbreeding with offspring and to mix genetic lines.

B. Keep each unique founder group together indefinitely and allow them to interbreed without mixing with other groups. This strategy is best for populations that have disease, husbandry, or logistical issues that would prohibit movement between groups.

C. Split the starting founder population in half and follow both strategies A and B (above) to increase chances of breeding success

****Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| < 1 year | 2 - 5 years |

**Example Species: *Eleutherodactylus, Nectophrynoides*, some *Hyperoliidae***

**Population Management Issue:** These species will lose genetic diversity fast. When managed in groups, larger population sizes are needed to ensure an adequate effective population size.

## INDIVIDUAL MANAGEMENT

- Individual Management may not be feasible for these types of species. However, if you choose to manage individually use the group management recommendations below to ensure long-term viability of the population.

## GROUP MANAGEMENT

**How many founders to collect?**

- You want 25.25 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 50.50 specimens.) Try to gather as even a sex ratio as possible.
- Keep founders in groups as small as possible (e.g., in pairs) to give equal breeding opportunity to all founders. If founders are kept in larger groups, you may need more founders to ensure 25.25 breeders.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 2 years and an effective population size of 0.15.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 400 |
| 40 | 635 |
| 55 | 875 |
| 70 | 1110 |
| 85 | 1350 |
| 100 | 1585 |

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size as quickly as possible due to short lifespan (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

Group Size

- Keep group sizes as small as is effective for the biology of the species-if possible try to maintain eight separate groups.
- Equalize family size across groups by keeping clutch sizes as equal as possible.
- If successfully breeding individuals within groups can be identified, consider removing them from the group to allow other individuals to breed.

Group Breeding Strategies: There are several strategies to retain gene diversity in populations of group-living animals:

A. Once reproduction occurs, systematically transfer individuals among groups in a “round robin” manner. We recommend one or more of these methods:
   - Transfer about 5 individuals per generation – This number may need to be increased if mortality is high or fecundity is low.
   - Transfer all juveniles – Move all juveniles out of their natal group to establish new next-generation groups before they reach reproductive maturity.
   - Transfer all of one sex – Move all males (or females) from one group to the next group to avoid inbreeding with offspring and to mix genetic lines.

B. Keep each unique founder group together indefinitely and allow them to interbreed without mixing with other groups. This strategy is best for populations that have disease, husbandry, or logistical issues that would prohibit movement between groups.

C. Split the starting founder population in half and follow both strategies A and B (above) to increase chances of breeding success

****Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| 1 - 5 years | < 5 years |

**Example Species: some *Hylidae*, some *Hyperoliidae*, *Scaphiophryne***

**Population Management Issue:** These species have short reproductive life spans, so breeding opportunities can be lost if delayed. With relatively short generation time, large population sizes will still be needed.

## INDIVIDUAL MANAGEMENT

**How many founders to collect?**

- You want 10.10 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 20.20 specimens.) Try to gather as even a sex ratio as possible.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 3 years and an effective population size of 0.30.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 135 |
| 40 | 215 |
| 55 | 290 |
| 70 | 370 |
| 85 | 450 |
| 100 | 530 |

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size as quickly as possible (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

- Breed according to mean kinship strategy (Lacy 1995, Pollak et al. 2005).
- Breed founders as long as possible; try to maintain equal numbers of offspring from all founders.
- Include at least some trial breeding of captive-born animals to ensure that population can be maintained when founders are gone.
- It is not necessary to keep generations discrete if animals are individually tracked.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| 1 - 5 years | < 5 years |

## GROUP MANAGEMENT

**How many founders to collect?**

- You want 25.25 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 50.50 specimens.) Try to gather as even a sex ratio as possible.
- Keep founders in groups as small as possible (e.g., in pairs) to give equal breeding opportunity to all founders. If founders are kept in larger groups, you may need more founders to ensure 25.25 breeders.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 3 years and an effective population size of 0.15.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 265 |
| 40 | 425 |
| 55 | 590 |
| 70 | 740 |
| 85 | 900 |
| 100 | 1060 |

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size as quickly as possible (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

Group Size

- Keep group sizes as small as is effective for the biology of the species-if possible try to maintain eight separate groups.
- Equalize family size across groups by keeping clutch sizes as equal as possible.
- If successfully breeding individuals within groups can be identified, consider removing them from the group to allow other individuals to breed.

Group Breeding Strategies: There are several strategies to retain gene diversity in populations of group-living animals:
A. Once reproduction occurs, systematically transfer individuals among groups in a “round robin” manner. We recommend one or more of these methods:

- Transfer about 5 individuals per generation – This number may need to be increased if mortality is high or fecundity is low.
- Transfer all juveniles – Move all juveniles out of their natal group to establish new next-generation groups before they reach reproductive maturity.
- Transfer all of one sex – Move all males (or females) from one group to the next group to avoid inbreeding with offspring and to mix genetic lines.

OR

B. Keep each unique founder group together indefinitely and allow them to interbreed without mixing with other groups. This strategy is best for populations that have disease, husbandry, or logistical issues that would prohibit movement between groups.

OR

C. Split the starting founder population in half and follow both strategies A and B (above) to increase chances of breeding success.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| 1 - 5 years | 5 - 15 years |

**Example Species:** ***Dendrobatidae*, *Typhlonectes*, *Tylototriton*/*Echinotriton*, *Theloderma*, *Cynops*, *Leptodactylus*, *Ceratobatrachus*, *Mantella*, *Atelopus***

**Population Management Issue:** These species have life histories that often start to approximate those of typical larger vertebrates, and therefore population management strategies can often be more like that used for most birds and mammals. However, although genetic management becomes easier, there may be more of a risk of demographic failure for species maintained at smaller numbers.

## INDIVIDUAL MANAGEMENT

**How many founders to collect?**

- You want 10.10 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 20.20 specimens.) Try to gather as even a sex ratio as possible.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 6 years and an effective population size of 0.30.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 70* |
| 40 | 110 |
| 55 | 150 |
| 70 | 190 |
| 85 | 225 |
| 100 | 265 |

*Note that this target size is the minimum recommended to meet genetic goals, but may be too small to meet demographic goals. In general, a population size of 100 is often considered the minimum needed to meet demographic goals.

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size as quickly as possible (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

- Breed according to mean kinship strategy (Lacy 1995, Pollak et al. 2005).
- Breed founders as long as possible; try to maintain equal numbers of offspring from all founders.
- Include at least some trial breeding of captive-born animals to ensure that population can be maintained when founders are gone.
- It is not necessary to keep generations discrete if animals are individually tracked.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| 1 - 5 years | 5 - 15 years |

## GROUP MANAGEMENT

**How many founders to collect?**

- You want 25.25 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 50.50 specimens.) Try to gather as even a sex ratio as possible.
- Keep founders in groups as small as possible (e.g., in pairs) to give equal breeding opportunity to all founders. If founders are kept in larger groups, you may need more founders to ensure 25.25 breeders.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 6 years and an effective population size of 0.15.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 140 |
| 40 | 225 |
| 55 | 300 |
| 70 | 370 |
| 85 | 450 |
| 100 | 530 |

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size as quickly as possible (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

Group Size

- Keep group sizes as small as is effective for the biology of the species-if possible try to maintain eight separate groups.
- Equalize family size across groups by keeping clutch sizes as equal as possible.
- If successfully breeding individuals within groups can be identified, consider removing them from the group to allow other individuals to breed.

Group Breeding Strategies: There are several strategies to retain gene diversity in populations of group-living animals:
A. Once reproduction occurs, systematically transfer individuals among groups in a “round robin” manner. We recommend one or more of these methods:

- Transfer about 5 individuals per generation – This number may need to be increased if mortality is high or fecundity is low.
- Transfer all juveniles – Move all juveniles out of their natal group to establish new next-generation groups before they reach reproductive maturity.
- Transfer all of one sex – Move all males (or females) from one group to the next group to avoid inbreeding with offspring and to mix genetic lines.

OR

B. Keep each unique founder group together indefinitely and allow them to interbreed without mixing with other groups. This strategy is best for populations that have disease, husbandry, or logistical issues that would prohibit movement between groups.

OR

C. Split the starting founder population in half and follow both strategies A and B (above) to increase chances of breeding success.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| 1 - 5 years | > 15 years |

**Example Species: *Salamandra*, some *Ambystoma***

**Population Management Issue:** These species have life histories very much like those of the larger vertebrates. Population management would benefit from moving toward individual management, rather than group management, whenever feasible.

## INDIVIDUAL MANAGEMENT

**How many founders to collect?**

- You want 10.10 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 20.20 specimens.) Try to gather as even a sex ratio as possible.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 7 years and an effective population size of 0.30.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 60* |
| 40 | 95* |
| 55 | 125 |
| 70 | 160 |
| 85 | 195 |
| 100 | 230 |

*Note that this target size is the minimum recommended to meet genetic goals, but may be too small to meet demographic goals. In general, a population size of 100 is often considered the minimum needed to meet demographic goals.

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size in one generation (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

- Breed according to mean kinship strategy (Lacy 1995, Pollak et al. 2005).
- Breed founders as long as possible; try to maintain equal numbers of offspring from all founders.
- Include at least some trial breeding of captive-born animals to ensure that population can be maintained when founders are gone.
- It is not necessary to keep generations discrete if animals are individually tracked.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| 1 - 5 years | > 15 years |

## GROUP MANAGEMENT

**How many founders to collect?**

- You want 25.25 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 50.50 specimens.) Try to gather as even a sex ratio as possible.
- Keep founders in groups as small as possible (e.g., in pairs) to give equal breeding opportunity to all founders. If founders are kept in larger groups, you may need more founders to ensure 25.25 breeders.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 7 years and an effective population size of 0.15.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 115 |
| 40 | 185 |
| 55 | 250 |
| 70 | 320 |
| 85 | 390 |
| 100 | 455 |

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size in one generation (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

Group Size

- Keep group sizes as small as is effective for the biology of the species-if possible try to maintain eight separate groups.
- Equalize family size across groups by keeping clutch sizes as equal as possible.
- If successfully breeding individuals within groups can be identified, consider removing them from the group to allow other individuals to breed.

Group Breeding Strategies: There are several strategies to retain gene diversity in populations of group-living animals:
A. Once reproduction occurs, systematically transfer individuals among groups in a “round robin” manner. We recommend one or more of these methods:

- Transfer about 5 individuals per generation – This number may need to be increased if mortality is high or fecundity is low.
- Transfer all juveniles – Move all juveniles out of their natal group to establish new next-generation groups before they reach reproductive maturity.
- Transfer all of one sex – Move all males (or females) from one group to the next group to avoid inbreeding with offspring and to mix genetic lines.

OR

B. Keep each unique founder group together indefinitely and allow them to interbreed without mixing with other groups. This strategy is best for populations that have disease, husbandry, or logistical issues that would prohibit movement between groups.

OR

C. Split the starting founder population in half and follow both strategies A and B (above) to increase chances of breeding success.

****Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| **> 5 years** | **≤ 15 years** |

**Example Species:**

**Population Management Issue:** Very slow population growth when fecundity is low, and the possibility for replacements of the population with predominantly the progeny of one or a few pairings when fecundity is high, means that each paring and each individual is important to population success.

## INDIVIDUAL MANAGEMENT

**How many founders to collect?**

- You want 10.10 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 20.20 specimens.) Try to gather as even a sex ratio as possible.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 5 years and an effective population size of 0.30.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 80* |
| 40 | 130 |
| 55 | 175 |
| 70 | 225 |
| 85 | 270 |
| 100 | 320 |

*Note that this target size is the minimum recommended to meet genetic goals, but may be too small to meet demographic goals. In general, a population size of 100 is often considered the minimum needed to meet demographic goals.

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size in one generation (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

- Breed according to mean kinship strategy (Lacy 1995, Pollak et al. 2005).
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- Include at least some trial breeding of captive-born animals to ensure that population can be maintained when founders are gone.
- It is not necessary to keep generations discrete if animals are individually tracked.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| **> 5 years** | **≤ 15 years** |

## GROUP MANAGEMENT

**How many founders to collect?**

- You want 25.25 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 50.50 specimens.) Try to gather as even a sex ratio as possible.
- Keep founders in groups as small as possible (e.g., in pairs) to give equal breeding opportunity to all founders. If founders are kept in larger groups, you may need more founders to ensure 25.25 breeders.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 5 years and an effective population size of 0.15.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 160 |
| 40 | 255 |
| 55 | 350 |
| 70 | 445 |
| 85 | 540 |
| 100 | 635 |

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size in one generation (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

Group Size

- Keep group sizes as small as is effective for the biology of the species-if possible try to maintain eight separate groups.
- Equalize family size across groups by keeping clutch sizes as equal as possible.
- If successfully breeding individuals within groups can be identified, consider removing them from the group to allow other individuals to breed.

Group Breeding Strategies: There are several strategies to retain gene diversity in populations of group-living animals:
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- Transfer all of one sex – Move all males (or females) from one group to the next group to avoid inbreeding with offspring and to mix genetic lines.

OR

B. Keep each unique founder group together indefinitely and allow them to interbreed without mixing with other groups. This strategy is best for populations that have disease, husbandry, or logistical issues that would prohibit movement between groups.

OR

C. Split the starting founder population in half and follow both strategies A and B (above) to increase chances of breeding success.

****Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| **> 5 years** | **> 15 years** |

**Example Species:** *Cryptobranchus*, *Andrias*

**Population Management Issue:** Genetic diversity can be maintained with relatively small populations, but these small populations may be vulnerable to demographic collapse or loss due to local environmental catastrophes hitting one out of only a few facilities.

**INDIVIDUAL MANAGEMENT**

**How many founders to collect?**

- You want 10.10 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 20.20 specimens.) Try to gather as even a sex ratio as possible.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 10 years and an effective population size of 0.30.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 45* |
| 40 | 65* |
| 55 | 90* |
| 70 | 115 |
| 85 | 140 |
| 100 | 160 |

*Note that this target size is the minimum recommended to meet genetic goals, but may be too small to meet demographic goals. In general, a population size of 100 is often considered the minimum needed to meet demographic goals.

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size in one generation (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

- Breed according to mean kinship strategy (Lacy 1995, Pollak et al. 2005)
- Breed founders as long as possible; try to maintain equal numbers of offspring from all founders.
- Include at least some trial breeding of captive-born animals to ensure that population can be maintained when founders are gone.
- It is not necessary to keep generations discrete if animals are individually tracked.

| Age to Maturity | Reproductive Lifespan |
|---|---|
| **> 5 years** | **> 15 years** |

## GROUP MANAGEMENT

**How many founders to collect?**

- You want 25.25 founders to survive and breed. Collect more based on your estimated rate of survival and reproductive success. (For example, if you expect 50% of the collected animals to survive and reproduce, you should collect 50.50 specimens.) Try to gather as even a sex ratio as possible.
- Keep founders in groups as small as possible (e.g., in pairs) to give equal breeding opportunity to all founders. If founders are kept in larger groups, you may need more founders to ensure 25.25 breeders.

**What is the target population size?**

- Target population size is defined as the minimum population size needed to meet genetic goals. This genetic target size may differ from the target size needed to meet demographic, research, or reintroduction goals.
- Target size depends on program length (e.g., short-term versus long-term) and species generation time.
- Target size was estimated using a generation time of 10 years and an effective population size of 0.15.
- These target sizes were estimated to maintain 90% gene diversity for the length of the program.

| Length of Program (Years) | Minimum Genetic Target Population Size |
|---|---|
| ≤ 25 | 80* |
| 40 | 130 |
| 55 | 180 |
| 70 | 225 |
| 85 | 270 |
| 100 | 320 |

*Note that this target size is the minimum recommended to meet genetic goals, but may be too small to meet demographic goals. In general, a population size of 100 is often considered the minimum needed to meet demographic goals.

**How quickly should you grow the population to the target size?**

- Grow the founding population to the target size in one generation (or at least five offspring per founder).
- After reaching the target size, each year determine the number of offspring needed to maintain the population size.

**Who should breed?**

Group Size

- Keep group sizes as small as is effective for the biology of the species-if possible try to maintain eight separate groups.
- Equalize family size across groups by keeping clutch sizes as equal as possible.
- If successfully breeding individuals within groups can be identified, consider removing them from the group to allow other individuals to breed.

Group Breeding Strategies: There are several strategies to retain gene diversity in populations of group-living animals:
A. Once reproduction occurs, systematically transfer individuals among groups in a “round robin” manner. We recommend one or more of these methods:

- Transfer about 5 individuals per generation – This number may need to be increased if mortality is high or fecundity is low.
- Transfer all juveniles – Move all juveniles out of their natal group to establish new next-generation groups before they reach reproductive maturity.
- Transfer all of one sex – Move all males (or females) from one group to the next group to avoid inbreeding with offspring and to mix genetic lines.

OR

B. Keep each unique founder group together indefinitely and allow them to interbreed without mixing with other groups. This strategy is best for populations that have disease, husbandry, or logistical issues that would prohibit movement between groups.

OR

C. Split the starting founder population in half and follow both strategies A and B (above) to increase chances of breeding success.

## ADDITIONAL CONSIDERATIONS

- Manipulating husbandry to minimize adaptation to captivity
- Avoid intentional and unintentional selection
- Husbandry learning curve—when bringing in a new species, possibly start with both pairs and groups to figure out best way to get them to breed
- Multiple paternity in individually-managed populations
- Husbandry research
- Holding capacity
- Number of holding institutions involved in each species
- Duration of captive program
- Disease concerns
- Enforcement of recommendations
- Training for those involved in amphibian captive management at their institutions
- Data entry issues—most software was not built for amphibian life history
- Ever-changing amphibian taxonomy
- 'Rules of Thumb' that should be researched more fully
    - High Priority
        - Develop methods to sex amphibians
        - Group management modeling
        - Effectiveness and Implications for possibility of selection for resistance or immunity to the *Batrachochytrium dendrobatidis* (Bd) chytrid fungus
    - Intermediate Priority
        - Demographic stability and fluctuations because captive demography may be very different from wild
        - Prevalence of different reproductive issues: multiple paternity, parthenogenesis, sperm storage, etc.
    - Lower Priority
        - Need to develop a tissue bank to preserve specimens/genetic material
        - Modeling of Ne/N guidelines per tank
        - Natural history research
        - Re-create Griffith's salmon study, but with amphibians—inadvertent selection for captive adaptations

# APPENDIX A
## Explanations for Population Management Recommendations

Compiled by Kevin Willis, Minnesota Zoo

**Recommendation Question1: How many founders to collect?**

**Figure 1: The probability that a sample of N animals will contain at least one individual of each sex.** This assumes that the source population has a 50%/50% male/female sex ratio. Any group with a size of 5 animals or larger has a 90% chance of including at least one of each sex. This assumes random sampling of individuals and no sexually dimorphic behaviors. The equation is P =1-0.5^(N-1).

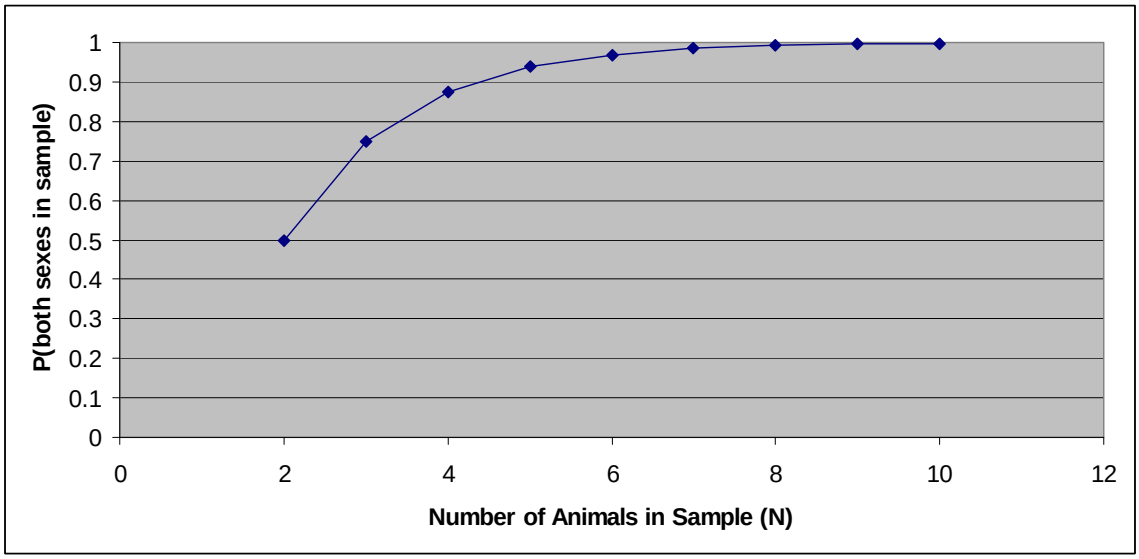
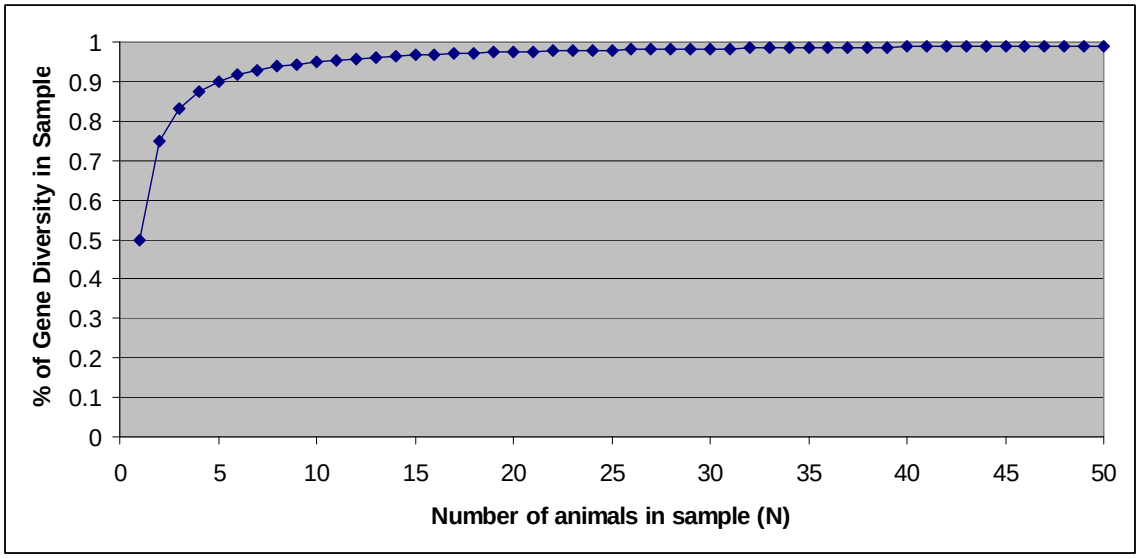
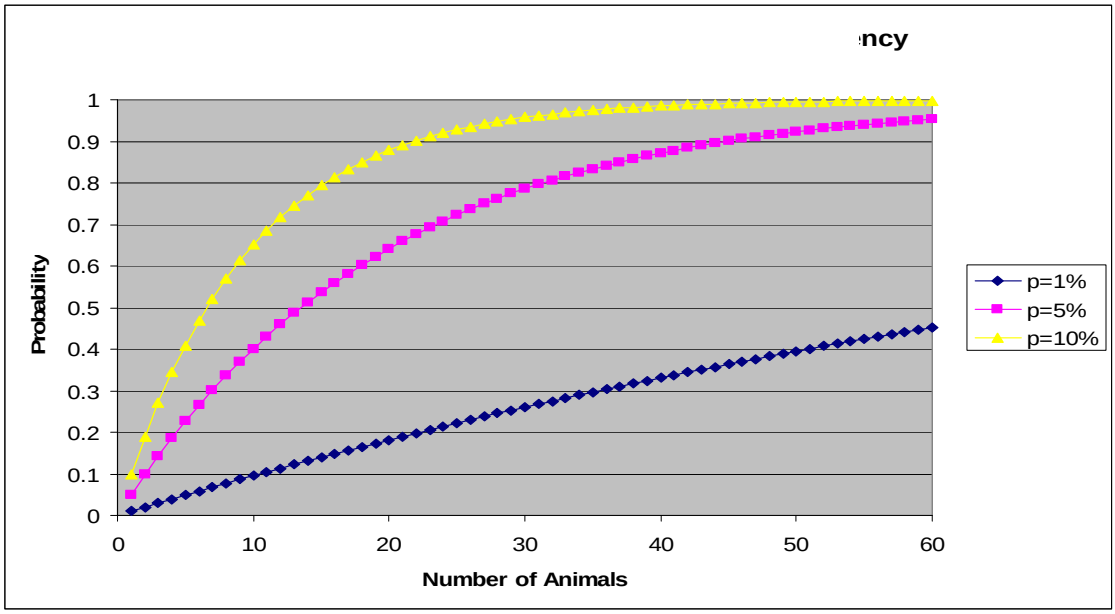



**Figure 2: The average percent of the gene diversity of the source population captured in a sample of N randomly selected animals.** This assumes the population is both homogeneous (i.e., no subpopulational structuring) and in Hardy-Weinberg equilibrium. Any number of founders larger than 20 will allow you to start with 97.5% potential gene diversity. The equation is GD = 1-1/(2N).



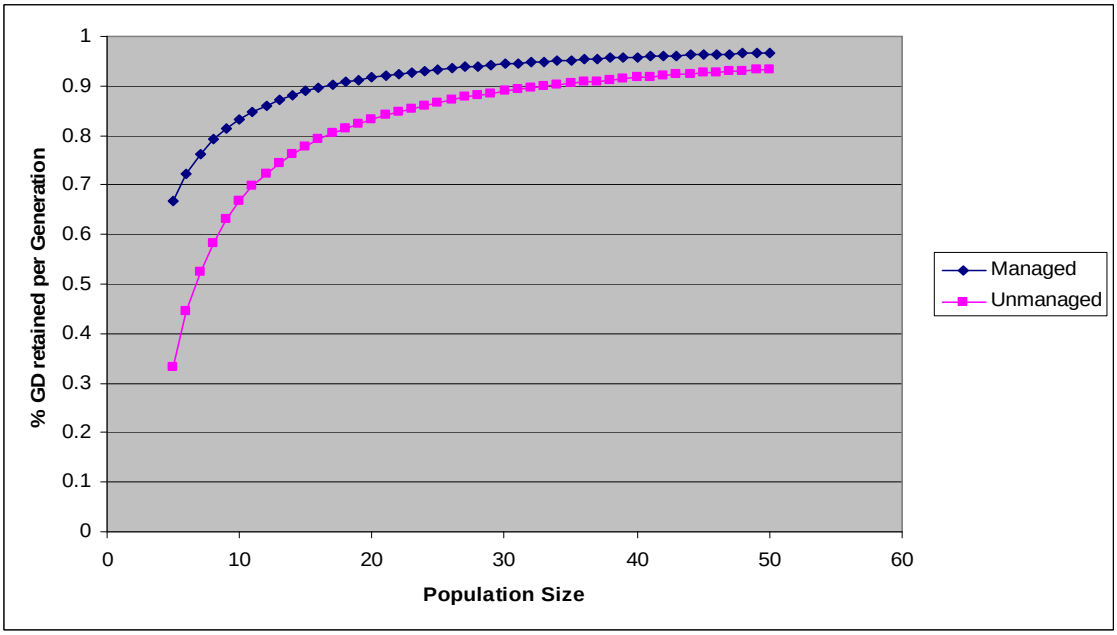

****Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

**Figure 3: Probability of obtaining an allele of a given frequency.** In addition to gene diversity, the probability that alleles are collected in a sample of N individuals is also of interest. This is a little more complex as the frequency of the allele is also a factor. In this figure the probability of obtaining an allele of frequency p in a sample of N randomly selected individuals is given for the values of p and samples sizes of 1 to 60 animals. The equation is 1 – (1-p)^N.




## Recommendation Question 2: What is the target population size?

**Figure 4: Gene diversity is lost on average each generation by an amount inversely proportional to the effective population size of the population.** The average rate of loss is 1/[2Ne] of the remaining gene diversity per generation, where Ne is the effective breeding population size, and factors such as the number of animals that produce offspring influence the relationship between the total population size and the effective population size. Shown here is a relationship between the population size (N) and the minimal rate of loss (in which Ne = 2 * N), a typical rate of loss (with Ne = 0.3 * N) for an intensively managed population, and a typical rate of loss (with Ne = 0.15 *N) for an unmanaged (group or random-breeding) population.




**Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

**Figure 5a: The number of generations until gene diversity drops below 90% for a population of size N with rates of loss as defined in figure 4.** Gene diversity is lost with each generation. The general equation is G=log(0.75)/log(1-1/(0.3*2N)) for an intensively managed population and unmanaged (group or random-breeding) population.

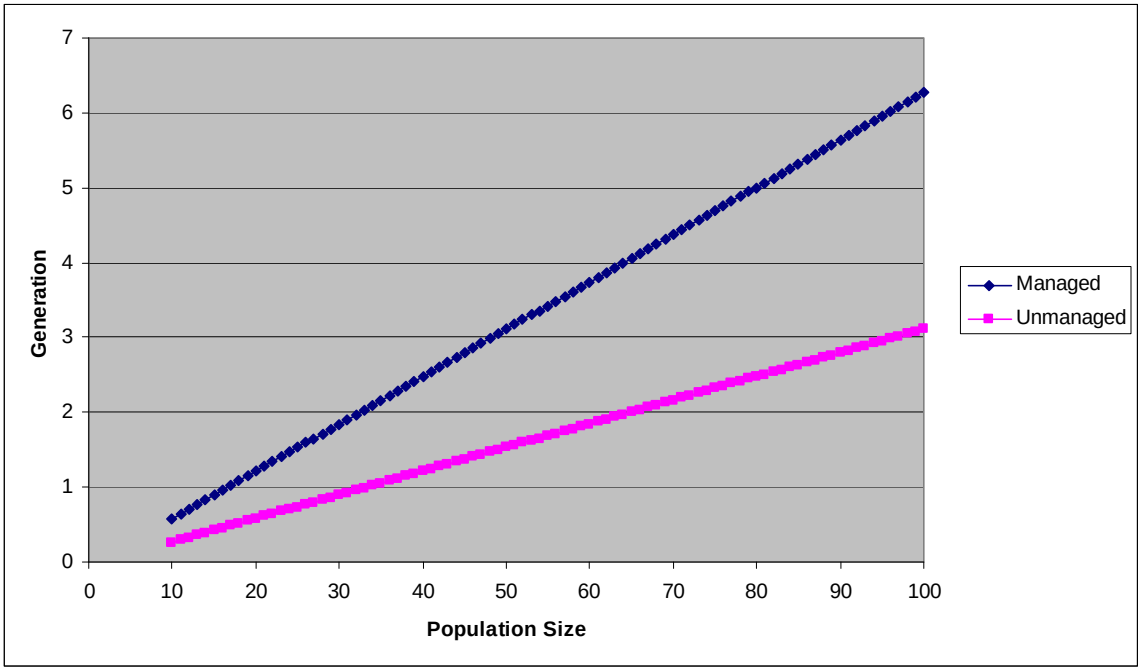
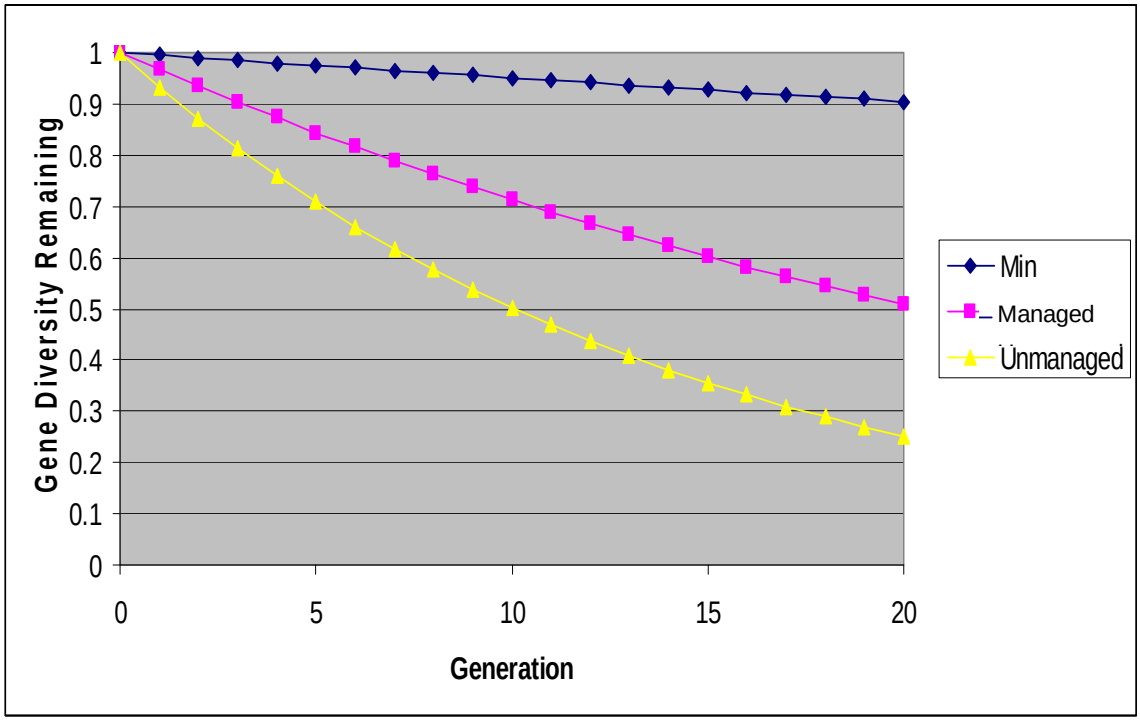



**Figure 5b: Proportion of gene diversity remaining for each generation for 20 generations based on a population of 50 animals with rates of loss of gene diversity as defined in figure 4.** Gene diversity is lost with each generation and gene diversity will be lost faster with less intensive management (as with groups). The general equation is GDt = (1-1/[4N])^t for an intensively managed population and unmanaged (group or random-breeding) population.
Note that this is an alternate view of the data in Figure 5a, but illustrates the same basic principle.




****Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

**Recommendation Question 3: How quickly should you grow the population to the target size?**

**Figure 6: Gene diversity remaining for each generation following the founding population.** The remaining gene diversity in generation t for a population of initial size X and target size N which grow at different rates. The formula is GD(t+1) = GDt*(1-1/2N)^(year/G);
where G = the generation interval, GDt is gene diversity at time t, GD(t+1) is not GD times (t+1) but rather GD at time t+1, and N is the effective population size. The graph is for populations with an effective size of 50, all starting with GD=1, and values of G = 2, 4 and 8.

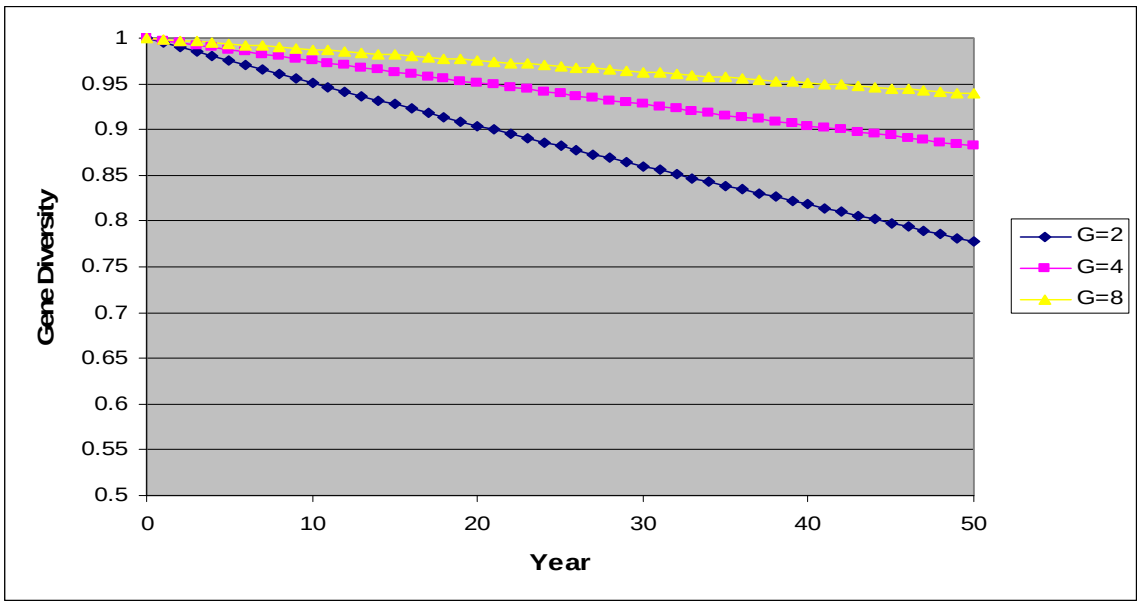
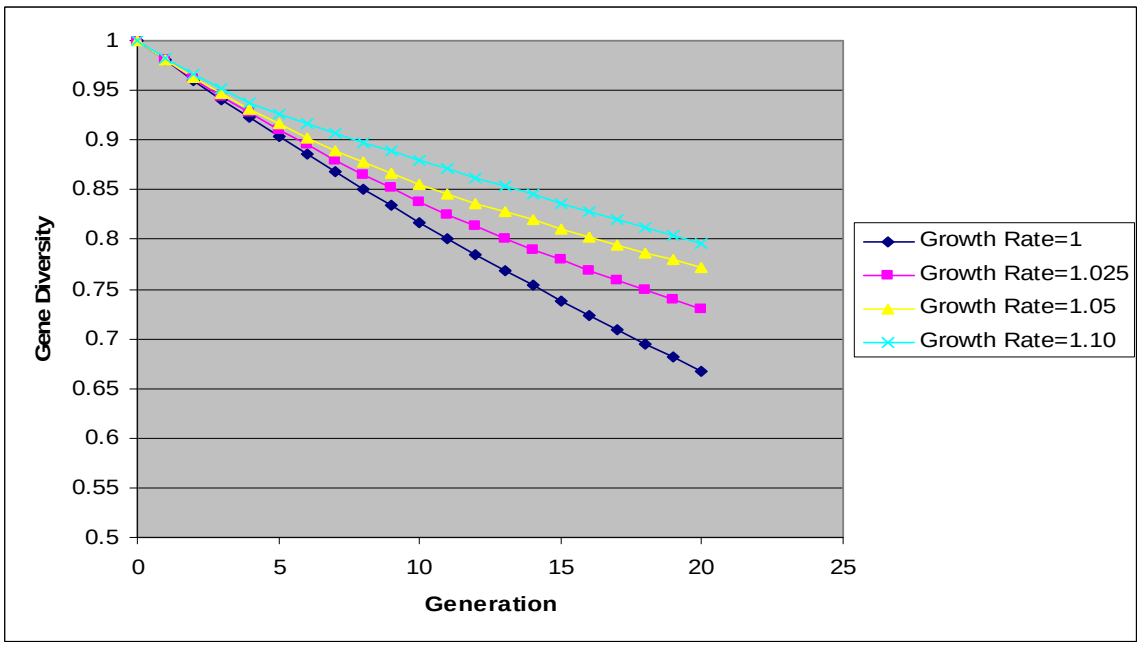



**Figure 7: Gene diversity is lost every generation and decreases more quickly with lower population growth rates.** In this example, each population starts with an effective size of 25 individuals and grows to a maximum effective size of 50 individuals. Each line represents a different growth rate per generation. The growth rate is multiplied by the number of effective individuals in this generation to determine the number of effective individuals in the subsequent generation and the rate of loss of gene diversity follows the standard drift equation.




**Please Note:** Throughout this document, the notation x.y will be used to signify x number of males and y number of females.

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*Chapter 7*

# Amphibian Data Entry Guidelines

Issued by:
Amphibian Ark
AZA Amphibian Taxon Advisory Group
AZA Institutional Data Management Advisory Group
AZA Population Management Center

23 September 2010

## INTRODUCTION

Amphibian life history characteristics can make data entry challenging. Large clutch sizes and an assortment of life stages can generate data entry inconsistencies within and among institutions and studbooks. As many AZA institutions currently hold amphibians, it is becoming clearer that these data can be recorded in a multitude of ways. The following guidelines will clarify amphibian data entry for both institutional registrars and studbook keepers.

Consistent recording of amphibian data entry can improve husbandry and population management for these populations in zoos and aquaria. Incomplete and inaccurate data can lead to inappropriate husbandry practices or management recommendations. Cooperative management among these institutions is the key to maintaining these populations. Current amphibian studbook keepers and institutional record keepers were surveyed for their current amphibian data conventions. This document is based on many of these currently used conventions. These are the first guidelines for amphibian data entry, so they may be used as a template or starting point for others around the world.

## CONTENTS

## USE OF GUIDELINES

The following amphibian data entry guidelines were created with both AZA registrars and studbook keepers in mind. Some Species Survival Plans (SSP), Population Management Plans (PMP), or conservation programs may have data conventions already in place, but amphibian data entry is not consistent across all institutions or populations. Institutional registrars create numerous amphibian-related reports (taxon, specimen, inventory, to permits, etc.) for SSP, PMP, and non-program amphibian populations, but because data entry is not standardized, it is difficult to compare reports. In all cases, it may be helpful for registrars and studbook keepers to be aware of the other's responsibilities in entering amphibian data.

This document was created as a reference for everyone working with amphibian data, no matter which software is used (ARKS4, Microsoft Access or Excel, PopLink, SPARKS, etc.). We expect that Program Leaders, Studbook Keepers, TAG Steering Committees, or institutions may come up with data entry conventions for their populations that reference this document and can be easily shared with others.

Each of the following Management Types provides a set of data entry protocols. Each protocol lists all acceptable options for recording the specific type of data listed. In some cases, there is only one option. However in others, the most ideal option is listed first and then followed by other options that are less ideal as far as data are concerned, but may involve less intensive data gathering techniques or staff time.

For example:

- Perhaps Amphibian Species A should use Protocols 1a, 2b, 3a, 4a, 5b, etc. because the individual amphibians are easily individually identifiable throughout their entire lives.
- In comparison, perhaps it would make the most sense for Amphibian Species B to use Protocols 10a, 11a, 12ai, etc. because it is a group managed species that cannot be individually identified.

## MANAGEMENT TYPES

Whether you use ARKS, Microsoft Access or Excel, SPARKS, PopLink, TRACKS, ZIMS, or your own in-house database, amphibian data for a particular species should ideally be entered consistently across institutions. Amphibian management, and in turn data entry, can be broken down into three types – Individually, as Groups, and Both Individually and as a Group.

I. Recorded '**Individually**' indicates that every animal is individually identifiable and has a unique ID, and/or is physically isolated for all life stages.

II. Recorded '**As Groups**' indicates that animals are identified with some type of group identification, not individually.

III. Recorded '**Both Individually and as a Group**' indicates that depending on life stage or other factors, some individuals or life stages may be individually marked and have unique IDs while other individuals or stages of an individual's life are recorded as part of a group. Often individuals in this situation are recorded as groups for the first part of their lives and then later individually identified once they reach a specific life stage.

**RECORD KEEPING FOR INDIVIDUAL MANAGEMENT –** every animal is individually identifiable and has a unique identity, and/or is physically isolated for all life stages.

**PROTOCOL 1:** Individual Identification

a. Identify and mark, if possible, using any reasonable method – transponders, photographs of physical characteristics, separate enclosures, etc. – to identify all individuals and assign an individual ID

**PROTOCOL 2:** Adding Individuals to Records
a. At hatch or live birth
b. Once they metamorphose, if applicable

**PROTOCOL 3:** Parentage of Individuals
a. Sire and dam should always be recorded, if possible
b. All potential sires and dams in enclosure at the time of conception should be recorded

**PROTOCOL 4:** Sex of Individuals
a. Sex should be recorded for each individual, if and when known

**PROTOCOL 5:** Birth / Hatch Date of Individuals
a. Date of egg hatch or live birth
b. Estimate if necessary

**PROTOCOL 6:** Individual vs. Clutch Birth / Hatch Dates
a. Each individual is given a specific birth/hatch date, regardless of when clutch mates hatch/are born
b. All clutch mates are given the same birth/hatch date

**PROTOCOL 7:** Transfer Events of Individuals
a. Only when moved to a new institution

**PROTOCOL 8:** Enclosure Composition for Individuals
a. At an institutional level, record which individuals live in the same enclosure (i.e., who is housed with whom) and record moves to/from enclosures containing the same species
b. At a studbook level, these data can be very important (e.g., can be used to determine potential parentage of offspring born/hatched in this enclosure), but there is not a specific location for entering this information in most studbook software. This could be entered as a User Defined Field (UDF) or Specimen Note.

**PROTOCOL 9:** Date and Cause of Death of Individuals
a. Add death event with date and location of death
b. Note cause of death (e.g., cause of natural death or a managed cull)

**PROTOCOL 10:** Additional Events, Activities, or Notes to Record for Individuals
a. Capture from wild
    i. Record site location, date of collection, number of individuals, sex ratio of individuals, life stage of individuals, and possible relationship to other wild caught individuals (i.e., several caught from the same location), if possible
b. Environmental changes (e.g., temperature, humidity, etc.)
c. Introduction of new animals into group (e.g., adding individual(s) of the same species or a different species to a group)
d. Link group records between institutions, if inconsistently identifying by group or individual
e. Merging groups (e.g., joining two or more groups to form one larger group)
f. Outbreaks of disease
g. Release to the wild
h. Splitting group (e.g., splitting one large group into two or more smaller groups)
i. Transferring group within an institution (i.e., moving them to a new enclosure, building, etc.)

**RECORD KEEPING FOR GROUP MANAGEMENT –** animals are identified with some type of group identification throughout their entire lives, never individually.

**PROTOCOL 11:** Group Identification

a. Assign an identification number to an each group; may be based solely on separate enclosures, etc.

**PROTOCOL 12:** Adding Offspring or Number of Offspring to Group Records

a. Add to records
    i. At hatch or live birth
    ii. Once they metamorphose, if applicable
b. Number of births/hatches in each group should be recorded
    i. Census initial number of offspring and number that make it to each life stage until adulthood
    ii. Census initial number of offspring number that make it to each life stage until they are split into different groups

**PROTOCOL 13:** Parentage of Groups

a. Sire and dam should always be recorded, if known
b. All potential sires and dams in enclosure at the time of conception should be recorded
    i. Studbook – record separate MULT# for each individual parent with a note indicating all potential sires and/or dams in enclosure (e.g., MULT1 = a few specific individuals that could be the potential sires, MULT2 = a different composition of animals that could be the potential dams)
    ii. Institutional Records – record any individual, group number, genetic material, or a possible parents note for potential sires and/or dams in enclosure
c. The number of males and females from within the group that are reproducing should be recorded whenever a new clutch is born/hatches
d. The number of reproductively mature individuals from within the group that could possibly be reproducing should be recorded whenever a new clutch is born/hatches

**PROTOCOL 14:** Birth / Hatch Dates within Groups

a. Date of egg hatch or live birth
b. Estimate if necessary

**PROTOCOL 15:** Clutch Birth / Hatch Dates within Groups

a. Each individual is given a specific birth/hatch date, regardless of when clutch mates hatch/are born (e.g., x number are born/hatched on one day, y number on the 2$^{nd}$ day, and z number on the 3$^{rd}$ day)
b. All clutch mates are given the same birth/hatch date

**PROTOCOL 16:** Generation Number, if possible

a. Number of generations from the wild (e.g., founder, F1, F2, etc.) should be recorded
b. Family lineages or family groups should be tracked

**PROTOCOL 17:** Group Census and Composition (who is housed with whom)

a. Sex ratio of group (i.e., number of males, females, unknown sex individuals) and number of individuals in each life stage (e.g., eggs, metamorphs, adults, etc.)
b. Numbers for each group should be recorded regularly, as determined by the Studbook Keeper, Program Leader, or Institution
    i. Weekly
    ii. Monthly
    iii. Yearly
    iv. Opportunistically (e.g., when exhibits are broken down or groups are moved)
c. Census Method
    i. Use exact census numbers whenever possible
    ii. If exact numbers are not possible, record estimated numbers with a note indicating the method of estimation

**PROTOCOL 18:** Additional Activities, Events, Notes, or Visits to Record for Groups

a. Death date and cause of death (i.e., indicate if death was due to various natural causes or a managed cull)
b. Developmental / life stage event
c. Capture from wild
    i. Record site location, date of collection, number of individuals, sex ratio of individuals, life stage of individuals, and possible relationship to other wild caught individuals (i.e., several caught from the same location), if possible
d. Environmental changes (e.g., temperature, humidity, etc.)
e. Group composition changes
    i. Introduction of new individuals into group (e.g., adding one or more individuals of the same species to a group or adding one or more individuals of a different species)
    ii. Merging groups (e.g., joining two or more groups to form one larger group)
    iii. Splitting a group (e.g., splitting one large group into two or more smaller groups)
f. Link group records between institutions, especially if inconsistently identifying by group or individual
    i. If a new institution records group members as individuals, record the group number in each individual record
    ii. If a new institution records individuals as a group, record all individual IDs in the group record
g. Outbreaks of disease
h. Release to the wild
i. Reproductive event, other than birth/hatch (e.g., amplexus or breeding behavior)
j. Transferring group to a new enclosure/tank

**RECORD KEEPING FOR BOTH GROUP AND INDIVIDUAL MANAGEMENT –** depending on life stage or other factors, some individuals or life stages may be individually marked and have unique IDs while other individuals or stages of an individual's life are recorded as part of a group. Often individuals in this situation are recorded as groups for the first part of their lives and then later individually identified once they reach a specific life stage.

**PROTOCOL 19:** Programs use different life stage milestones to change from recording a group to individual identification, but should be recorded individually as soon as possible and consistently for the entire population.

a. Reach froglet, toadlet, tadpole, or juvenile stage
b. Reach adult stage
c. When transpondered
d. When physically individually identifiable
e. When begin being housed separately

**PROTOCOL 20:** Some populations may have instances where individually identified amphibians are changed to group identified populations, but should be recorded individually as long as possible, but consistently for the entire population. Some examples of these instances are:

a. Individuals are no longer physically individually identifiable
b. Can no longer be housed separately
c. Individuals are moved to a new institution where they will be group managed
    i. If a new institution records group members as individuals, record the group number in each individual record
    ii. If a new institution records individuals as a group, record all individual IDs in the group record

**PROTOCOL 21:** Individual Institutional Identification after Medical Treatment

a. Only if an animal is individually identifiable or kept separately thereafter
    i. Could also be used to temporarily identify an individual due to separation, but will no longer be, or need to be, individually identifiable once added back into the group
b. Add a brief explanation about the affected individual in the group text entry

For additional protocols for those species with both individual and group management, see previous sections on individual and group management that could also apply to these amphibian species.

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**AUTHORED BY**

Kristine Schad, AZA Population Management Center & Amphibian Ark, kschad@lpzoo.org

With the help of:

Diane Barber, Fort Worth Zoo & Amphibian Taxon Advisory Group
Nanette Bragin, The Denver Zoo & Institutional Data Management Advisory Group
Tim Carpenter, Seattle Aquarium
Shelly Grow, Association of Zoos & Aquariums
Adrienne Miller, International Species Information System (ISIS) & Institutional Data Management Advisory Group
Jean Miller, Buffalo Zoo & Institutional Data Management Advisory Group
R. Andrew Odum, Toledo Zoological Society & Institutional Data Management Advisory Group
Andy Snider, Fresno Chaffee Zoo & Institutional Data Management Advisory Group
Amphibian Taxon Advisory Group Steering Committee (ATAG)
Institutional Data Management Advisory Group Steering Committee