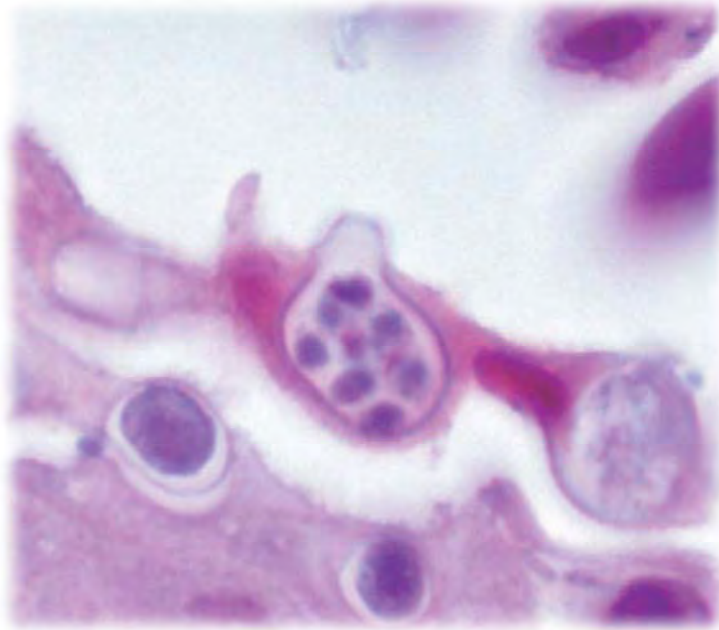


A Manual For Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs

Proceedings from a Workshop:
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San Diego Zoo



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A contribution of the IUCN/SSC Conservation Breeding Specialist Group in collaboration with Amphibian Ark, San Diego Zoo, and Zoo Atlanta

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A MANUAL FOR CONTROL OF INFECTIOUS DISEASES IN AMPHIBIAN SURVIVAL ASSURANCE COLONIES AND REINTRODUCTION PROGRAMS

EDITORS: ALLAN P. PESSIER & JOSEPH R. MENDELSON III

**PROCEEDINGS FROM A WORKSHOP:
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Zoological Society of San Diego**

Organized by:

Allan P. Pessier, DVM, Dipl. ACVP
San Diego Zoo
&
Joseph R. Mendelson III, PhD
Zoo Atlanta

Facilitated by:

Onnie Byers, PhD
IUCN Conservation Breeding Specialist Group

Table of Contents

1. INTRODUCTION.....	8
2. LIST OF CONTRIBUTORS.....	10
3. RISK ASSESSMENT.....	11
3.0 INTRODUCTION.....	11
3.1 GENERAL RECOMMENDATIONS FOR REINTRODUCTION AND TRANSLOCATION PROGRAMS.....	11
3.2 DISEASE RISK MITIGATION FOR CAPTIVE BREEDING AND REINTRODUCTION PROGRAMS.....	13
3.3 TRANSLOCATION.....	19
3.4 REFERENCES.....	20
4. BIOSECURITY.....	21
4.0 INTRODUCTION.....	21
4.1 WHAT IS "BIOSECURITY".....	22
4.2 BIOSECURITY DEFINITIONS.....	23
4.3 COSMOPOLITAN COLLECTIONS AND RISKS OF DISEASE FOR WILD AMPHIBIANS... ..	23
4.4 LEVELS OF BIOSECURITY.....	24
4.5 SUMMARY OF BEST PRACTICES AND ISOLATION SECURITY LEVELS.....	30
4.6 BIOSECURITY PRACTICES.....	33
4.7 STAFF TRAINING AND IMPLEMENTATION OF BIOSECURITY PRACTICES.....	33
4.8 LONG-TERM ISOLATION OF AMPHIBIANS DESTINED FOR REINTRODUCTION TO THE WILD.....	35
4.9 FACILITIES FOR LONG-TERM ISOLATION.....	36
4.10 HUSBANDRY STAFF HYGIENE AND PROTECTIVE CLOTHING.....	37
4.11 HUSBANDRY ROUTINES.....	39
4.12 ENCLOSURE SANITATION.....	40
4.13 EQUIPMENT, ENCLOSURE SUBSTRATES AND DECORATION.....	41
4.14 WASTEWATER DISPOSAL.....	42
4.15 SOLID WASTE DISPOSAL.....	44
4.16 SOURCES OF WATER.....	45
4.17 SOURCES OF FOOD.....	45
4.18 FACILITY DESIGN AND BUILDING SPECIFICATIONS.....	46
4.19 REFERENCES.....	47
5. CLEANING AND DISINFECTION.....	50
5.0 INTRODUCTION.....	50
5.1 PRINCIPLES OF CLEANING & DISINFECTION.....	51
5.2 CHEMICAL DISINFECTANTS.....	53
5.3 NON-CHEMICAL METHODS OF DISINFECTION.....	56

5.4	CLEANING AND DISINFECTION OF ANIMAL ENCLOSURES.....	57
5.5	DISINFECTION OF EQUIPMENT AND TOOLS.....	58
5.6	DISINFECTION AND STERILIZATION OF SUBSTRATES AND CAGE FURNITURE.....	59
5.7	DISINFECTION OF LIVE PLANTS.....	60
5.8	DISINFECTION AND STERILIZATION OF WATER.....	61
5.9	DISINFECTION AND BIOSECURITY IN THE FIELD.....	62
5.10	TABLE OF DISINFECTANTS.....	64
5.11	REFERENCES.....	66
6.	QUARANTINE.....	69
6.0	INTRODUCTION.....	69
6.1	GOALS AND DEVELOPMENT OF QUARANTINE PROGRAMS.....	69
6.2	RISK ASSESSMENT IN AMPHIBIAN QUARANTINE.....	71
6.3	QUARANTINE FACILITY AND QUARANTINE STAFF CONSIDERATIONS.....	72
6.4	ANIMAL HUSBANDRY CONSIDERATIONS IN QUARANTINE.....	74
6.5	LENGTH OF THE QUARANTINE PERIOD.....	78
6.6	MEDICAL CONSIDERATIONS IN QUARANTINE.....	80
6.7	LABORATORY TESTING AND DISEASE TREATMENT IN QUARANTINE.....	83
6.8	NECROPSY.....	84
6.9	TESTING AND TREATMENT FOR INTERNAL PARASITES.....	85
6.10	TESTING AND TREATMENT FOR THE AMPHIBIAN CHYTRID FUNGUS.....	87
6.11	TESTING FOR <i>RANAVIRUS</i>	91
6.12	CLINICAL PATHOLOGY (HEMATOLOGY AND CLINICAL BIOCHEMISTRY).....	94
6.13	OTHER INFECTIOUS DISEASES ENCOUNTERED IN QUARANTINE.....	95
6.14	REFERENCES.....	99
7.	DIAGNOSTIC TESTING.....	102
7.0	INTRODUCTION.....	102
7.1	DISEASE SURVEILLANCE.....	103
7.2	EVALUATION OF LABORATORIES THAT PERFORM MOLECULAR DIAGNOSTIC TESTING.....	107
7.3	DIAGNOSTIC TESTING FOR THE AMPHIBIAN CHYTRID FUNGUS (<i>BATRACHOCHYTRIUM DENDROBATIDIS</i>).....	111
7.4	<i>RANAVIRUS</i> INFECTION.....	125
7.5	<i>PERKINSUS</i> -LIKE PROTOZOAL DISEASE OF RANID FROGS.....	135
7.6	DISEASE REPORTING AND PARTICIPATION IN DISEASE DATABASES.....	137
7.7	TESTING AND SURVEILLANCE FOR FECAL PARASITES.....	138
7.8	HEMATOLOGY AND SERUM BIOCHEMISTRY.....	148
7.9	LABORATORIES THAT ACCEPT AMPHIBIAN SAMPLES.....	149
7.10	REFERENCES.....	151
8.	TREATMENT AND CONTROL OF DISEASES.....	159
8.0	INTRODUCTION.....	159
8.1	TREATMENT AND CONTROL METHODS FOR THE AMPHIBIAN CHYTRID FUNGUS.....	160

8.2 CONTROL OF <i>RANAVIRUS</i> INFECTIONS.....	171
8.3 CREATING SPECIFIC-PATHOGEN-FREE AMPHIBIAN POPULATIONS.....	173
8.4 PARASITE MONITORING AND TREATMENT.....	178
8.5 ELECTROLYTE FORMULAS.....	181
8.6 EUTHANASIA.....	182
8.7 REFERENCES.....	182
9. NECROPSY.....	186
9.0 INTRODUCTION.....	186
9.1 NECROPSY PREPARATION.....	187
9.2 MATERIALS NEEDED.....	187
9.3 PERFORMING THE NECROPSY.....	188
9.4 ANATOMICAL NOTES FOR AMPHIBIAN NECROPSIES.....	192
9.5 SAMPLE COLLECTION DURING MORTALITY EVENTS.....	195
9.6 SHIPMENT OF SAMPLES.....	196
9.7 REFERENCES.....	197
APPENDIX 1: METHODS TO INDIVIDUALLY IDENTIFY THE AMPHIBIAN SPECIMEN.....	198
APPENDIX 2: WASTEWATER TREATMENT.....	217
APPENDIX 3: ISOLATED AMPHIBIAN ROOMS AT OMAHA’S HENRY DOORLY ZOO.....	221

CHAPTER 1

INTRODUCTION

The International Union for the Conservation of Nature (IUCN) has estimated that 30% of all amphibian species are threatened with extinction, and at least 484 species are Critically Endangered (<http://www.iucnredlist.org>). The major contributing factor of the most drastic amphibian population declines is the disease chytridiomycosis caused by the chytrid fungus *Batrachochytrium dendrobatidis*. This fungus, disseminated worldwide by anthropogenic means, can reduce amphibian biodiversity at new locations in alarmingly short periods of time. Thus, understanding and controlling infectious diseases such as chytridiomycosis have become a major focus of both *in situ* and *ex situ* amphibian conservation efforts worldwide.

To formulate a response to the crisis of global amphibian extinctions, the Species Survival Commission (SSC) of the IUCN convened an international group of leading amphibian biologists at an Amphibian Conservation Summit resulting in the Amphibian Conservation Action Plan (ACAP; this document is available for download at <http://www.amphibians.org>). The ACAP concluded that the amphibian extinction crisis “requires a global response at an unprecedented scale from governments, corporations, civil society and the scientific community.” The ACAP calls for creation of survival assurance colonies that bring representatives of critically endangered amphibian species into captivity for safekeeping. Although remediation of factors contributing to species extinction is preferable to captivity for endangered animals, the rapidity of amphibian declines makes it likely that innumerable species will be lost long before suitable solutions are discovered. Therefore, the critical role of survival assurance colonies is to preserve the option of re-introducing species to their native habitat at a later time. The global effort to develop such programs is coordinated by the Amphibian Ark, a joint effort of the World Association of Zoos and Aquariums (WAZA), the IUCN/SSC, Conservation Breeding Specialist Group (CBSG) and Amphibian Specialist Group (www.amphibianark.org). The Amphibian Ark estimates 500 amphibian species require immediate *ex situ* intervention through establishment of survival assurance colonies. This is a massive undertaking that calls upon the resources of a wide variety of institutions including zoos, aquariums, botanical gardens, natural history museums, governmental agencies, and universities. Yet, as institutions prepare for the “call to action” from the Amphibian Ark and IUCN, there is a global reality that the field of amphibian husbandry in general is still very much in its infancy and expertise in amphibian disease control is limited to a very few institutions worldwide.

In response to urgent needs created by the rapid expansion of amphibian survival assurance colonies worldwide, guidelines for Best Practices for permanent quarantine and facility biosecurity were drafted as part of the CBSG/WAZA Amphibian

Ex Situ Conservation Planning Workshop held in El Valle, Panama, in 2006 (proceedings available at <http://www.amphibianark.org/downloads.htm>). In addition, the final report of the Panama workshop outlined additionally needed Action Steps to develop medical protocols for health screening, prophylactic treatment of important infectious diseases and disease surveillance in captive amphibian collections. Elements of these Action Steps have already emerged since 2006 amidst a flood of new information in the primary literature on amphibian disease testing, disease control protocols, and the development of creative approaches to meeting strict biosecurity guidelines within existing *ex situ* institutional facilities and culture. The present manual aims to formally address these Action Steps by bringing together recommendations from husbandry experts and veterinarians with expertise in amphibian medicine and disease risk assessment to consolidate new information into simple consensus-based protocols that can be easily adapted by the wide variety of institutions that maintain captive amphibians.

This manual is intended to serve as a “one-stop shopping” resource for basic consensus based recommendations concerning amphibian quarantine, necropsy, facility biosecurity, facility hygiene & disease treatment, and disease screening & surveillance. These are to be viewed as guidelines and recommendations and not as mandates or requirements; no simple manual can take into account the specifics of individual programs and needs. Importantly, this manual is not intended as a replacement for a complete text on amphibian veterinary medicine (e.g., *Amphibian Medicine and Captive Husbandry*, by K. M. Wright & B. R. Whitaker, Malabar Publ., 2001). Similarly, although the topics of nutrition and basic husbandry are discussed throughout, this manual is intended merely as a complement to existing texts on amphibian husbandry, such as the *Amphibian Husbandry Resource Guide, Edition 1.0* (Association of Zoos and Aquariums Publ., 2008) or species-specific protocols such as that prepared for the Panamanian golden frog (<http://www.ranadorada.org/species-info.html>). This manual is intended to be a detailed technical resource for veterinarians and senior staff in *ex situ* or conservation programs, to guide them as they develop new or additional amphibian programs, design routine screening protocols or encounter infectious disease issues in current collections, or move animals among collections or reintroduce them into the wild. This manual will be iterative, with subsequent editions made available following an additional workshop planned for 2011, and occasionally thereafter.

We hope users from all backgrounds find this manual to be a useful primary resource and, importantly, a convenient portal into the primary literature on amphibian veterinary science and diseases. The amphibians of the world are in need of considerable assistance to avoid additional extinctions, and we hope this manual expedites some of that needed assistance.

Allan P. Pessier
Joseph R. Mendelson III
25 December, 2009

CHAPTER 2

LIST OF PARTICIPANTS

The following persons contributed directly to the final content that appears in this version of the manual. Most of them participated in the workshop, held at San Diego Zoo in February, 2009, and added important comments to drafts of various chapters as they neared completion.

Diane Barber, Fort Worth Zoo
Eric Baitchman; Zoo New England
Don Boyer, San Diego Zoo
Onnie Byers, IUCN Conservation Breeding Specialist Group
Leigh Ann Clayton, National Aquarium Baltimore
Josh Cook, University of Georgia
Ann Duncan, Detroit Zoo
Ryan DeVoe, North Carolina Zoo
Shannon Ferrell, Fort Worth Zoo
Ron Gagliardo, Amphibian Ark & Zoo Atlanta
Michael Garner, Northwest ZooPath
Richard Gibson, Amphibian Ark & Chester Zoo
Andrea Johnson, San Diego Zoo
Megan Jones, San Diego Zoo
Kim Lovich, San Diego Zoo
Bob Mailloux, Sandfire Dragon Ranch
Denise McAloose, Wildlife Conservation Society-Bronx Zoo
Joseph Mendelson, Zoo Atlanta
Pat Morris, San Diego Zoo
Isamara Navarrete, San Diego Zoo
R. Andrew Odum, Toledo Zoo
Brad Lock, Zoo Atlanta
Samuel Rivera, Zoo Atlanta
Allan Pessier, San Diego Zoo
Jennifer Pramuk, Wildlife Conservation Society-Bronx Zoo
Michael Ready, Sandfire Dragon Ranch
Oliver Ryder, San Diego Zoo
Danna Schock, University of Calgary
Mark Schrenzel, San Diego Zoo
Lee Skerratt, James Cook University
Edythe Sonntag, Michigan State University
Simone Vitali, Perth Zoo
Carmel Witte, San Diego Zoo
Kevin Wright, Arizona Exotic Animal Hospital
Kevin Zippel, Amphibian Ark

Chapter 3

RISK ASSESSMENT AND DISEASE SCREENING FOR AMPHIBIAN REINTRODUCTION PROGRAMS

3.0 INTRODUCTION

The recognition that many devastating global amphibian population declines may be related to anthropogenic movement of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, highlights the need to seriously consider infectious diseases in the operation of amphibian captive breeding and reintroduction programs. An important lesson that can be learned from the example of amphibian chytridiomycosis is that all facilities that keep captive amphibians for any purpose (e.g., education, commerce, laboratory research and conservation) should take responsibility for implementing infectious disease surveillance and control measures that prevent the introduction of amphibian pathogens to new locations or populations (see Section 4. 3).

A recently published report describes the introduction of the amphibian chytrid fungus to wild populations of the Mallorcan Midwife Toad as the result of a species reintroduction program (Walker et al., 2008). Additionally many biologists anecdotally recall instances where amphibian disease may have been moved to new locations as the result of reintroductions or well-meaning translocation of wild animals from one location to another. In many of these cases simple disease surveillance or prevention methods could have been invaluable in preventing disease introduction.

This chapter provides an overview of disease risk assessment, surveillance and control practices that can be applied to amphibian captive breeding, reintroduction and translocation programs in order to reduce disease risks. Every amphibian conservation program will be unique and absolute recommendations that will apply to every circumstance are not possible. Instead, examples are given of common scenarios encountered by workshop participants and it is hoped that this will provide a conceptual framework for the development of new programs and practices. While this chapter has a purposeful focus on chytridiomycosis and ranaviral diseases, our community must constantly remain observant and vigilant with respect to as-yet undocumented or unsuspected pathogens.

3.1 GENERAL RECOMMENDATIONS FOR REINTRODUCTION AND TRANSLOCATION PROGRAMS

Maintaining amphibians in captive breeding programs prior to reintroduction to the wild or during translocation of wild animals from one location to another have inherent risks for the introduction of infectious diseases to new locations or populations.

- It is impossible to create programs that are free of disease risk.
- Each program needs to determine what level of risk is acceptable to its stakeholders (e.g., government wildlife authorities; wildlife biologists; veterinarians; zoo program curators; among others).
- In some situations, the disease risk of a reintroduction or translocation is so high that important programmatic decisions may need to be reconsidered (e.g., a decision is made not to reintroduce animals because the disease risk is too high).
- Fortunately, the disease risk of reintroduction or translocations is significantly reduced by careful planning and adherence to recommendations that help to mitigate risk.

The concerns about the risk of introducing infectious diseases to wild populations as the result of species reintroduction and translocation programs are not limited to amphibians. To address these concerns, tools have been developed to guide veterinarians and animal managers through a process of disease risk assessment.

- The IUCN/SSC Conservation Breeding Specialist Group has published an *Animal Movements and Disease Risk* workbook that is available for free download at: www.cbsg.org/cbsg/content/files/Disease_Risk/disease.risk_manual.pdf

Suggestions for disease risk mitigation that are specific for amphibian programs and discussed at the Disease Control Workshop include:

- When possible amphibian reintroduction or translocation programs should be operated within the native range of the species. Programs that keep amphibians outside of the native range of the species (e.g., amphibians from Panama kept in the United States) have higher disease risks than programs located within the native range of the species. These increased risks include both the risks of introducing non-native pathogens into the environment around the facility holding the amphibians, and the possibility of introducing novel local pathogens to the imported amphibians (see Section 4. 3).
- Amphibians used in translocation or reintroduction programs should be kept in “Long Term Isolation” from amphibians that are from outside of the native range of the species that will be reintroduced. This process of isolation is especially important for zoos that want to participate in amphibian conservation programs, but that have amphibian collections that are mixed or “cosmopolitan” (e.g., amphibians from multiple geographic locations are housed in one facility). Details on the process of permanent isolation are found in Section 4. 7). In the case of translocations, the time that amphibians spend in captivity should be minimized.
- Reintroduction and translocation programs should collect background disease and health-related information on the captive population that will be reintroduced as well as the wild population (and sympatric amphibian species) that already exist in the area where reintroductions will occur. Knowledge of the disease problems

occurring in both populations allows for better assessment of the disease risk posed by release of captive animals.

- Methods that are used to develop a database of health information for captive and wild populations include:
 - Necropsy (including histopathology) of all animals that die (see Chapter 9). Necropsy is valuable for detecting new or unsuspected infectious diseases that are not detected by specific tests such as PCR for the amphibian chytrid fungus.
 - Necropsy surveillance is not limited to animals that die naturally and can also include sacrifice (culling) of amphibians from populations to obtain optimal samples for laboratory investigation.
 - Targeted testing of captive and wild populations for specific amphibian pathogens (e.g., the amphibian chytrid fungus or ranaviruses) as needed (see Chapter 7).
- Disease monitoring of populations is performed prior to release as well as after animals have been reintroduced. All mortality events in the wild population should be investigated and documented.
- Additional targeted disease testing (e.g., amphibian chytrid fungus or ranaviruses) may be necessary prior to release of captive animals to the wild or prior to translocation.

3.2 DISEASE RISK MITIGATION FOR CAPTIVE BREEDING AND REINTRODUCTION PROGRAMS

Programs that bring amphibians from threatened wild populations into captive survival assurance populations for captive breeding and eventual release of progeny back into the wild are an important amphibian conservation strategy. Strategies for disease risk mitigation and disease screening of populations prior to release of animals back into the wild are discussed for low, intermediate and high risk situations below:

Low-Risk Situations

The lowest risk situation for introduction of a significant infectious disease to wild amphibian populations as the result of a reintroduction program is when survival assurance populations are located within the native country of the species or species assemblage AND the facility keeps only amphibians from inside the native country. Examples of in-country amphibian survival assurance populations are the El Valle Amphibian Conservation Center (EVACC) in Panama and the Balsa de los Sapos amphibian conservation program at the Catholic University (Pontificia Universidad Católica del Ecuador) in Ecuador.

Risk mitigation strategies:

1. Facilities are located in the native range of the species or species assemblage and only keep amphibians from the native range.

2. Facilities maintain “Best Practices” of biosecurity. These are discussed in detail in Section 4.5).
3. A quarantine program is in place for new animals that enter the facility (see Chapter 6).
4. Major morbidity and mortality events are investigated by necropsy examination and histopathology (see Chapter 9). Routine examination of all, or most, deaths is recommended to build a health-history database and to have the best chance of detecting unsuspected infectious disease problems.
5. Development of specific-pathogen free (e.g., amphibian chytrid fungus or *Ranavirus*) captive populations should be considered (see Section 8.3).
6. Animals are released only in the native range of the species (preferably original source location)

Disease testing and treatment prior to release into the wild:

Animals that are reared only in the native range AND are never exposed to animals from outside the native range usually have minimal requirements for disease testing prior to release into the wild. With some exceptions, pathogens present in these animals are presumed to be those present in the wild population. Guidelines for designing pre-release disease testing and treatment protocols for these populations include:

1. If an infectious disease problem is identified during routine necropsy and histopathology of the captive population the following is recommended:
 - If a treatable pathogen is identified (e.g. the amphibian chytrid fungus), treatment is recommended if pathogen can be eliminated (see Chapter 8).
 - Treatment is most important if the pathogen is known to cause significant morbidity and mortality in the captive population (e.g., amphibian chytrid fungus).
 - A reliable testing method must be available to confirm that animals are free of infection prior to release to the wild.
 - Some parasites/pathogens are difficult to eliminate or it is desirable to maintain low levels of infection with organisms that occur naturally in wild populations (especially parasites). See Section 8.4.
 - Define the potential impact of that pathogen on the wild population. This is achieved through ongoing disease surveillance of wild population and other susceptible species.
 - If the identical pathogen is present in both the captive and wild populations, the presence of the pathogen may not impact the decision to release captive animals to the wild. Determination if pathogens are identical is not always straightforward. For

instance, there are many species of ranaviruses that appear identical on routine PCR screening methods. Advanced laboratory methods are required to determine the species of *Ranavirus* present (see Section 7.4).

- Some amphibian pathogens are opportunistic pathogens (e.g., water molds or mycobacteria) and are usually diseases of captive husbandry rather than a high disease risk for wild populations.
2. Consider pre-release testing for pathogens known to be significant to wild amphibian populations (e.g., amphibian chytrid fungus and ranaviruses).
 - Testing may not be necessary if a comprehensive captive population health history is available that includes necropsy and histopathology surveillance of the majority of animals that die (as noted above) with no indication that deaths due to chytridiomycosis or *Ranavirus* infection have occurred. Surveillance should have been conducted over an extended period of time (at least 1–2 years). See Section 8.3 for details on creation of specific-pathogen free (SPF) amphibian populations.
 3. Individual animals that are sick or members of a captive population experiencing a mortality event should never be released into the wild until after identification and resolution of the illness. This is true even if the pathogen is known to occur in the wild population.

Moderate-Risk Situations

A moderate-risk situation for introduction of a significant infectious disease to wild amphibian populations as the result of a reintroduction program is when the captive can be exposed to amphibians that originate from outside the native range of the species. This occurs at facilities either within the range country (e.g., a facility working with German species in Germany, but also keeps amphibians from Asia or the United States) or when survival assurance populations are located outside of the range country (e.g., German amphibians kept in the United States).

Risk mitigation strategies:

1. Facilities maintain amphibian species that will be reintroduced to the wild in “Long Term Isolation” (see Section 4.7).
2. A quarantine program is needed for new animals that enter the facility (see Chapter 6).
3. Major morbidity and mortality events are investigated by necropsy examination and histopathology (see Chapter 9). Routine examination of all or most deaths is suggested to build a health-history database (see Section 6.2) and to have the best chance of detecting unsuspected infectious disease problems.

4. Development of specific-pathogen-free (e.g., amphibian chytrid fungus or *Ranavirus*) captive populations should be considered (see Section 8.3).

Disease testing and treatment prior to release into the wild:

In moderate risk situations, guidelines for designing pre-release disease testing and treatment protocols for these populations include:

1. If an infectious disease problem is identified during routine necropsy and histopathology of the captive population the following is recommended:
 - If a treatable pathogen is identified (e.g. amphibian chytrid fungus), treatment is recommended if pathogen can be eliminated (see Chapter 8).
 - Treatment is most important if the pathogen is known to cause significant morbidity and mortality in the captive population (e.g., amphibian chytrid fungus).
 - A reliable testing method must be available to confirm that animals are free of infection prior to release to the wild.
 - Some parasites/pathogens are difficult to eliminate or it may be desirable to maintain low levels of infection with organisms that occur naturally in wild populations (especially parasites). See Section 8.4
 - Define the potential impact of that pathogen on the wild population. This is achieved through ongoing disease surveillance of wild population and other susceptible species.
 - If the identical pathogen is present in both the captive and wild populations, the presence of the pathogen may not impact the decision to release captive animals to the wild. Determination if pathogens are identical may not be straightforward. For example, there are many species of ranaviruses that appear identical on routine PCR screening methods. Advanced laboratory methods are required to determine the species of *Ranavirus* present. See Section 7.4.
 - Some amphibian pathogens are opportunistic (e.g., water molds or mycobacteria) and are more often diseases of captive husbandry rather than a high disease risk for wild populations.
2. Testing for pathogens known to be significant to amphibian populations (e.g., amphibian chytrid fungus and ranaviruses) should be performed prior to release into the wild. Culling of a subset of animals for specific pathogen testing may be helpful. Specific testing may not be necessary if a comprehensive captive population health history is available that includes necropsy and histopathology surveillance of the majority of animals that die (as noted above) with no indication that deaths due to chytridiomycosis or *Ranavirus* infection have occurred. Surveillance should have

been conducted over an extended period of time (at least 1–2 years). Ideally, these populations would be known as specific-pathogen free (see Section 8.3).

3. Individual animals that are sick or members of a captive population experiencing a mortality event should never be released into the wild until after identification and resolution of the illness. This is true even if the pathogen is known to occur in the wild population.

High-Risk Situations

A high-risk situation for introduction of a significant infectious disease to wild amphibian populations as the result of a reintroduction program is 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 (See Chapter 4).

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 of reintroducing animals to the wild.

Risk mitigation strategies:

1. Place animals into conditions of in “Long-Term Isolation” (see Section 4.7). Consider releases only after multiple generations of animals are bred under isolation conditions.
2. A quarantine program is needed for new animals that enter the facility (see Chapter 6).
3. Major morbidity and mortality events are investigated by necropsy examination and histopathology (see Chapter 9). Routine examination of all or most deaths is suggested to have the best chance of detecting unsuspected infectious disease problems and for development of a comprehensive group health history. The group health history should be collected over an extended period of time with a minimum of 1–2 years of data collection.
4. Develop specific-pathogen free captive populations for the amphibian chytrid fungus and ranaviruses (see Section 8.3).
5. Consider creating a new population of animals that is derived by removal of eggs or embryos from breeding of the original captive population. This may be an important

measure for reducing the risk from novel pathogens that are not easily detected by routine testing.

6. Recognize that if disease mitigation strategies are not effective, then animals should not be reintroduced to the wild under any circumstances.

Disease testing and treatment prior to release into the wild:

The level of disease screening needed for the release of animals from High-Risk situations back into the wild can be extensive.

1. If an infectious disease is identified during routine necropsy and histopathology of the captive population the following is recommended:
 - If a treatable pathogen is identified (e.g. amphibian chytrid fungus), treatment is recommended if pathogen can be eliminated (see Chapter 8).
 - Treatment is most important if the pathogen is known to cause significant morbidity and mortality in the captive population (e.g., amphibian chytrid fungus).
 - A reliable testing method must be available to confirm that animals are free of infection prior to release to the wild.
 - Define the potential impact of that pathogen on the wild population. This is achieved through ongoing disease surveillance of wild population and other susceptible species.
 - If the identical pathogen is present in both the captive and wild populations, the presence of the pathogen may not impact the decision to release captive animals to the wild. Determination if pathogens are identical may not be straightforward. For example, there are many species of ranaviruses that appear identical on routine PCR screening methods. Advanced laboratory methods are required to determine the species of *Ranavirus* present (see Section 7.4).
 - Some amphibian pathogens are opportunistic (e.g., water molds or mycobacteria) and are more often diseases of captive husbandry rather than a high risk for wild amphibian populations.
2. If infectious diseases are identified that are not easily treated or eliminated, a new population of animals (re-derivation) should be created by:
 - Removing eggs from the original source population and hatching tadpoles in permanent isolation away from the source population. For species that are viviparous (e.g., Kihansi spray toad) investigate the feasibility of caesarian section and rearing of tadpoles in permanent isolation.
 - Re-derivation of multiple generations with serial passage and rearing of these generations into new permanent isolation environments may be necessary.

- Disinfection of eggs or egg masses prior to rearing in permanent isolation can be considered if a method can be identified that does not interfere with development or viability of tadpoles.
3. Perform experiments where captive animals for release are housed with sympatric amphibian species present in the location or region where releases will occur.
 - Sympatric animals are monitored for disease development and necropsies with histopathology are performed on all animals that die.
 - Animals surviving until the end of the experiment are euthanized. Necropsies and histopathology are performed on animals at this time.
 - At the time of necropsy, collect tissues from all animals for use in diagnostic tests for pathogens of interest (e.g., save samples of liver and kidney frozen for *Ranavirus* PCR).
 - Experiments should be conducted for at least 60–90 days to allow for manifestation of any significant infectious diseases.
 4. Culling and euthanasia of a subset of the captive population for necropsy and histopathology as well as testing for specific pathogens of interest and concern (e.g., PCR for ranaviruses) prior to release into the wild is necessary.
 5. Review a 1–2 year health history of the population to be released into the wild that includes necropsy and histopathology reports from all or most animals that die. Do not release animals into the wild that have evidence of an infectious disease process.

3.3 TRANSLOCATION

Translocations occur when wild animals that are moved between different locations with little or no intervening time in captivity (e.g., movement between ponds or canyons; animals that are part of same metapopulation). Some disease risks in translocations include:

- Movement of a significant amphibian pathogen (e.g., the amphibian chytrid fungus) from one location to another
- Acquisition of pathogens from captive amphibians (if animals are held in captivity during the process of translocation)

Risk mitigation strategies:

1. If animals are held in captivity prior to translocation they should be maintained in long-term isolation from other captive or wild amphibians (see Section 4.7). Isolation biosecurity practices should be in place during the entirety of time in transportation and captivity.

2. Investigate deaths during translocation utilizing necropsy examination and histopathology (see Chapter 9) to identify unsuspected disease problems.
3. If possible, prior to translocation perform health surveillance studies of both the source and destination wild populations. This can include necropsy and histopathology surveillance as well as targeted testing for important pathogens such as the amphibian chytrid fungus and ranaviruses. These studies will define the disease risks of the translocation.
4. If infectious agents are identified, define the potential impact of that pathogen on the wild population.
 - If the identical infectious agent is present in both the source and destination populations, the presence of the pathogen may not impact the decision to perform the translocation. Determination if pathogens are identical may not be straightforward. For instance, there are many species of ranaviruses that appear identical on routine PCR screening methods. Advanced laboratory methods are required to determine the species of *Ranavirus* present (see Section 7.4).
 - If the infectious agent is present only in the source population. Additional planning and risk assessment are necessary prior to translocation.
 - Consideration may be given to a short period of captivity for treatment of treatable infectious diseases (e.g., chytridiomycosis) in the source population. Treatment may not be necessary or desired if the same pathogen is present in both the source and destination populations.

3.4 REFERENCES

Walker, S. F., J. Bosch, T. Y. James, A. P. Litvintseva, J. A. Oliver-Valls, S. Piña, G. Garcia, G. A. Rosa, A. A. Cunningham, S. Hole, R. Griffiths, and M. C. Fisher. 2008. Invasive pathogens threaten species recovery programs. *Current Biology* 18:R853–R854.

CHAPTER 4

BIOSECURITY AND PERMANENT ISOLATION OF *EX SITU* CONSERVATION POPULATIONS**4.0 INTRODUCTION**

The example of chytridiomycosis as an infectious disease introduced worldwide by anthropogenic means and resulting in devastating amphibian population declines has highlighted a need for improved biosecurity in facilities that keep captive amphibians. Amphibians are routinely moved globally for use as laboratory research subjects, as pets, as educational or display animals and as part of conservation and breeding programs. These movements can increase the risk that amphibian pathogens will be moved to new locations as has been demonstrated in recent studies of amphibian imports and movements (Fisher and Garner, 2007; Schloegel et al., 2009; Schloegel et al., 2010) and by the documented introduction of the amphibian chytrid fungus to wild populations of the Mallorcan midwife toad by the activities of a captive breeding program (Walker et al., 2008).

Implementation of biosecurity practices that reduce the potential for introduction of amphibian infectious diseases to new locations are the responsibility of all institutions that maintain or move captive amphibians. In addition, good biosecurity practices help to reduce the risk posed by infectious diseases on the success and sustainability of captive amphibian programs.

The major concepts of amphibian biosecurity are:

- There are risks of infectious disease associated with programs that keep amphibians in captivity outside of the native geographic range of the species or species assemblage (e.g., Panamanian frogs brought to the United States). Similar risks occur anytime animals from multiple geographic regions are mixed in one captive amphibian facility (a mixed or cosmopolitan collection).
- The simplest and least expensive way to reduce these risks is to maintain captive amphibians only within the native range of the species and to avoid creation of cosmopolitan amphibian collections.
- If captive amphibians must be kept outside of their native range or within a cosmopolitan collection biosecurity practices that reduce the risk of disease transmission are necessary.
- Captive breeding programs or survival assurance colonies that intend to reintroduce amphibians to the wild should maintain those animals in permanent isolation (e.g., dedicated rooms or buildings) away from amphibians that originate from outside the native range of the species. Husbandry practices such as the use of dedicated footwear, protective clothing, dedicated tools and equipment, and following specific work-flow patterns reduce the risk of introducing non-native pathogens to amphibian collections. Facilities located within the natural range of the species and that do not keep amphibians from

outside the natural range are the best example of permanent isolation and require the least amount of expense and effort.

- Use of relatively simple husbandry routines and practices reduces the risk of introducing and spreading infectious diseases within an amphibian facility. This is regardless of the role of the species (e.g., education, pet, or as a survival assurance population).
- Procedures for disposal of solid waste and amphibian facility wastewater are considered whenever captive amphibians are held outside the native range of the species or whenever captive amphibians are housed in a cosmopolitan collection that keeps amphibians from different geographic regions (inside or outside the native range).
- Sources for food and water in the facility are scrutinized for the potential to introduce amphibian pathogens.
- Ideally, facilities are: pest-proof; amphibian-proof (for escape of captives from the facility or for entry of or contact with free-ranging native amphibians); designed for automation in feeding, watering and cleaning; and easy to clean and maintain.
- Ideally, facilities provide for the unique environmental needs of amphibians in regard to environmental temperature, humidity, lighting, and water quality. These criteria are essential to amphibian health and are the subject of several expert reviews (Browne et al., 2007; Pramuk and Gagliardo, 2008),

4.1 WHAT IS "BIOSECURITY"?

Biosecurity can be defined as "the protection of the environment and its native species from exotic pathogens." However, the management of *ex situ* populations of amphibians for conservation purposes requires additional considerations. Therefore, in this document biosecurity refers to measures that:

- Protect native species in the natural environment from pathogens that are carried by captive amphibians (especially if captive species are held outside of their natural range or are exposed to other amphibians from outside their natural range).
- Protect captive specimens from pathogens present in the native amphibians (whether facility is in-range or out-of-range).
- Protect captive specimens from pathogens present in other captive amphibians in the collection.

We will never achieve 100% biosecurity in any *ex situ* amphibian population.

- The opportunities for pathogen movement are significantly reduced by identifying vectors and husbandry practices that present potential risks and designing protocols to remove or reduce these risks.
- Implementing and maintaining appropriate biosecurity is a never ending process of risk assessment (i.e., probability of pathogen transfer occurring by any identified vector/practice weighed against the likely severity of the consequences) and subsequent risk reduction.

A realistic level of appropriate biosecurity can be achieved with protocols that are simple and inexpensive to put into practice. Significant investment in facilities and equipment is only required to achieve the highest level of biosecurity—for example, when working outside of the natural range of a species intended for eventual reintroduction to the wild.

4.2 BIOSECURITY DEFINITIONS

Program located inside range of species: The facility (or facilities) holding the amphibian species or assemblage of species is physically located inside the geographic distribution of the species. *Examples:* 1) A building or modified shipping container with Kihansi spray toads (*Nectophrynoides asperginis*) located at the rim of Kihansi Gorge, Tanzania; 2) A collection of multiple species of locally captured amphibians housed in an educational center for visitors inside a national park or natural reserve.

Program located outside range of species: The facility (or facilities) holding the amphibian species or assemblage of species is physically located outside the geographic distribution of the species. *Examples:* 1) Kihansi spray toads (*Nectophrynoides asperginis*) located in a zoo in the USA; 2) wild-caught salamanders from southern Mexico (e.g., Chiapas State) being maintained in a facility in central Mexico (e.g., Michoacan State).

Cosmopolitan facility or institution: Any facility (e.g., room or building) or institution (e.g., university or zoo) that maintains species of amphibians from different geographic regions—i.e., species that do not co-occur in the wild. The reality is that most amphibian programs in the world qualify as “cosmopolitan” but this does not mean that they cannot develop adequately secure programs toward amphibian conservation and reintroductions.

Isolated facility or institution: Any facility (e.g., room or building) or institution (e.g., university or zoo) that is dedicated to only a single species or assemblage of co-occurring amphibian species. These sorts of programs usually are focused on a single species, such as at the US Fish & Wildlife Service Wyoming toad facility (*Anaxyrus baxteri*) at Saratoga National Fish Hatchery. In some restrictive cases, this concept may be applied to separate populations, rather than actual species.

4.3 COSMOPOLITAN COLLECTIONS AND RISKS OF DISEASE FOR WILD

AMPHIBIANS

Pathogens that are native (or endemic) to one population of amphibians often cause only a mild or even undetectable illness in those animals. This is because the population has been exposed to the pathogen over time and has developed adaptations to limit the pathogen’s harmful effects. However, when introduced to new (naïve) amphibian populations the same “non-native” pathogen has the potential to cause more severe disease.

Traditional zoo amphibian collections typically are “cosmopolitan”, keeping animals from different geographic locations and different sources (e.g., wild-caught; captive-born; obtained from a commercial animal dealership) all within the same facility. These collections increase the risk of introducing non-native amphibian pathogens to naïve species or geographic locations because:

- Direct or indirect contact between animals in cosmopolitan collections can facilitate the transmission of pathogens between animals from different locations (e.g., frogs from Central America are exposed to frogs from Australia and become infected with a pathogen that is native only to frogs from Australia).
- If animals infected with a “non-native” pathogen are later reintroduced to the wild they will also introduce the “non-native” pathogen to a new location (e.g., Central American frogs infected with an Australian pathogen are reintroduced to the wild in Central America).
- Amphibians brought to a new geographic location can introduce non-native amphibian pathogens to native amphibians in the new location (e.g., frogs from Central America infected with a pathogen native to Central America are brought to the United States).

For these reasons, efforts should be made to ensure that programs that keep amphibians in captivity are not contributing to the movement of amphibian pathogens.

The simplest and least expensive ways to reduce the disease risk of moving “non-native” amphibian pathogens to new locations are to:

- Establish and maintain captive (*ex situ*) amphibian populations within, or as close as possible to, the native range and habitat of the species that is the subject of a conservation program (e.g., maintain Honduran species in Honduras and Australian species in Australia).
- Avoid establishing captive breeding programs of amphibians intended for reintroduction to the wild inside a cosmopolitan amphibian collection (within the natural range of the species or outside the natural range of the species).

Because this is not always possible, measures to increase biosecurity are necessary for many captive amphibian programs.

4.4 LEVELS OF BIOSECURITY

Biosecurity measures are specific husbandry, staff work-flow and veterinary procedures that reduce, but do not completely eliminate, infectious disease risks from within an amphibian facility.

The reasons for implementation of biosecurity practices in captive amphibian facilities are to:

- Prevent “non-native” amphibian pathogens from leaving a captive facility and becoming introduced to new locations. This can occur when amphibians have been removed from their native range for any purpose.

- Prevent amphibian pathogens from entering a captive population or from being transmitted between animals within a facility. For example, it is not desirable to introduce the amphibian chytrid fungus into a healthy amphibian collection or to spread infection with the fungus throughout an amphibian collection.

The types of biosecurity practices needed to achieve these goals is determined individually for each amphibian facility and for each amphibian conservation program. In cosmopolitan amphibian collections, biosecurity practices are determined for each species kept in the facility.

In this manual, guidelines for two levels of biosecurity are defined based on the degree and types of infectious disease risks that are identified in a captive amphibian program.

- The basic protocol is “**BEST PRACTICES**” which requires neither special equipment nor facilities, but outlines simple common-sense practices for hygiene and prevention disease spread for ALL captive amphibians.
- For *ex situ* populations that are intended for eventual use in reintroduction programs, a few extra biosecurity measures are required to reduce risks of introducing pathogens into the wild. This protocol is termed simply “**ISOLATION**”, as the simple act of physically separating a population of animals greatly reduces the majority of risks of disease spread among captive animals.

The decision to use either BEST PRACTICES or ISOLATION protocols is aided by a simple risk assessment and decision tree (see below).

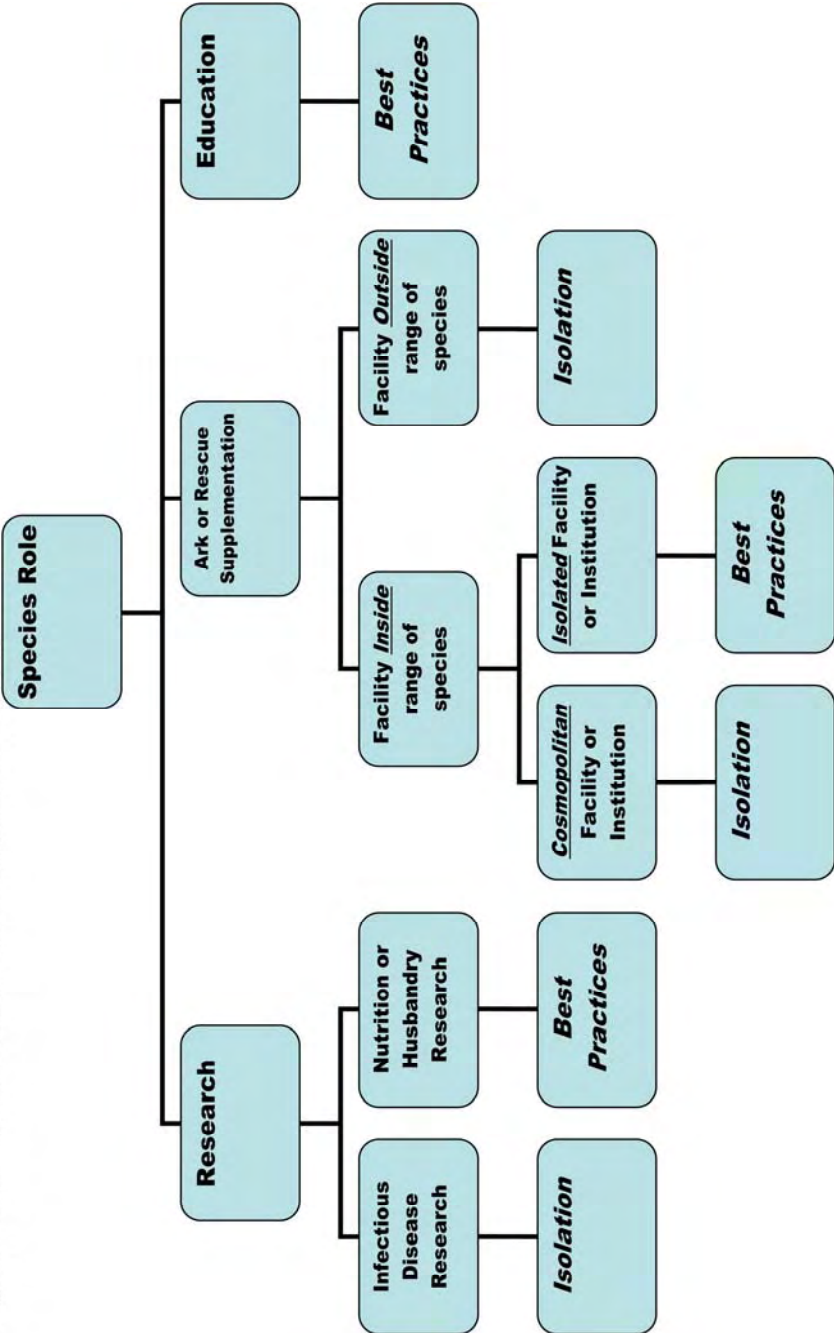
Risk Assessment Decision Tree for Biosecurity Information Required

The decision tree for biosecurity risk assessment (Figure 4.1) requires three types of information:

1. The role of a species or species assemblage in a conservation program
2. The location of the amphibian facility in relation to the geographic origin of the species or species assemblage
3. Is the captive facility “cosmopolitan” or “isolated” (see Section 4.2)

These criteria are explained in detail below (after the Decision Tree).

Figure 4.1: Decision tree for biosecurity risk assessment.



1. The 'role' of the species or species assemblage

The most common species roles for captive amphibians, as defined by the CBSG/WAZA *Ex Situ* Amphibian Planning Workshop (Zippel, Lacy and Byers 2006) and by Amphibian Ark are listed below, with the addition of a category for large-scale commercial operations.

Roles for Captive Amphibians:

- Ark, Rescue or Supplementation
- Conservation Research
- Conservation Education
- Amphibian Farming and Mass Production

Ark, Rescue, or Supplementation:

All of these categories have the intention of reintroducing the animals or their progeny to the wild.

Ark—An amphibian species that is extinct in the wild (locally or globally) and which would become completely extinct without *ex situ* management.

Rescue—An amphibian species that is in imminent danger of extinction (locally or globally) and requires *ex situ* management as part of the *recommended* conservation action.

Supplementation—An amphibian species for which *ex situ* management benefits the wild population through breeding for release as part of the *recommended* conservation action.

Conservation Research:

These animals have no prospect of reintroduction to the wild, but are used for specific applied research projects that contribute to the conservation of that species, or a related (surrogate) species, in the wild.

Examples of this kind of research include:

- Development of techniques for captive breeding.
- Disease research (e.g., control or treatment of chytridiomycosis).
- Nutrition and development of captive amphibian diets.

Conservation Education:

These animals have no prospect of return to the wild. These are amphibian species that are used only for educational purposes—primarily in zoos and aquariums—to inspire and increase knowledge of visitors, in order to promote positive behavioral change. These animals are those typically held in a cosmopolitan zoo collection but may also include amphibians that are “flagship” or “ambassador” species used to raise awareness of amphibian conservation issues (e.g., an endangered Wyoming toad removed from the captive breeding population to be used as a display animal).

Occasionally, amphibians used for educational purposes are released back into the wild. If this is the case, the animals should be considered as Ark/Rescue/Supplementation for purposes of the risk assessment.

Amphibian Farming and Mass Production:

These animals are reared intensively and in large numbers for food, use as laboratory animals, and for the pet trade. This group of animals has unique disease risks because of the sheer number of animals housed, the common use of outdoor enclosures (allows for escape of animals and contact between native amphibians and farmed amphibians) and the potential for transmission of pathogens in cosmopolitan species situations (e.g., a amphibian or reptile dealership that sells animals from different geographic regions).

2. The location of the amphibian facility

The location of the facility holding captive amphibians is a very important component of the biosecurity risk assessment. The most important considerations are the following dichotomies:

- The facility is within the natural geographic range of the species or species assemblage or near the site of original collection.
- OR**
- The facility is outside of the natural range of the species or species assemblage, or distant from site of original collection.

3. Is the facility cosmopolitan or isolated?

- The facility is limited to keeping species or species assemblages from within the natural geographic range (isolated).
- OR**
- The facility maintains species or species assemblages from outside the natural range (e.g., cosmopolitan zoo collection).

Examples Using the Risk Assessment Decision Tree

The following scenarios are provided to guide readers in the use of the Risk Assessment Decision Tree for Biosecurity (Figure 4.1).

Example 1:

American bullfrogs (*Lithobates catesbeianus*) housed in a zoo exhibit in the United States that educates the public about wetland ecosystems. There are no plans to breed this species in captivity or release offspring back into the wild.

Using this information in the decision tree:

- These bullfrogs are not part of a captive breeding or conservation research program and they (or their offspring) will never be released back into the wild. Therefore, have an EDUCATION role.
- Animals with an EDUCATION species role are managed in captivity using the BEST PRACTICES level of biosecurity.

- The BEST PRACTICES biosecurity guidelines (see Section 4.4) include information on how to prevent introduction of infectious diseases carried by the bullfrogs into local wild amphibian populations to or other amphibians that are in the zoo collection. This would also be true if the exhibit mentioned in this example were located outside of the United States (e.g. Europe or Australia), however, considerable scrutiny of biosecurity practices for waste and wastewater disposal (see Sections 4.14–4.15) and aspects of facility design related to preventing escape of captive animals or entry of native wild amphibians (see Section 4.18) is warranted because of significant risks associated with the introduction of “non-native” amphibian pathogens to new locations (see Section 4.3 above).

Example 2:

Wyoming Toads (*Anaxyrus baxteri*) are an endangered species from the western United States that are the subject of a captive breeding program that produces tadpoles for reintroduction into the wild. One facility in the captive breeding program is a zoo located 20 miles (32 km) from a lake where the toad is known to naturally occur. The zoo is a mixed cosmopolitan institution that also keeps a small number of other amphibian species from around the world for educational purposes.

Using this information in the decision tree:

- The Wyoming toads are part of a captive breeding program that aims to reintroduce animals back into the wild. Therefore, these toads have an Ark, Rescue or Supplementation role.
- The zoo in this example is inside the native range of the Wyoming Toad.
- The zoo in this example is a “mixed” facility or institution that also keeps other amphibian species from outside the natural range of the Wyoming Toad.
- Animals with a Ark, Rescue or Supplementation role that are housed in a “mixed” facility or institution are kept in an ISOLATION level of biosecurity. In this situation this is true even though the animals are kept in a facility that is within the native range of the toad. Additional precautions in the ISOLATION biosecurity level (see Section 4.4) help to ensure that toads released to the wild have not been exposed to “non-native” pathogens that circulate in other amphibians kept in the mixed facility.

A second facility that houses Wyoming Toads for the captive breeding program is located 15 miles (24 km) from a lake where the toad is known to naturally occur. This facility is dedicated to breeding only Wyoming toads and no other amphibians are kept in this facility.

- Because the facility in this example is within the native range of the Wyoming Toad and because the facility is dedicated (or “Isolated”) to the Wyoming Toad, the animals can be kept under the BEST PRACTICES level of biosecurity (see Section 4.4). The BEST PRACTICES biosecurity level still includes “common sense” recommendations to reduce the risk that important amphibian pathogens (e.g. the amphibian chytrid fungus) are not introduced or spread in the captive population.

Example 3:

Kihansi Spray Toads (*Nectophrynoides asperginus*) are extinct in the wild in their native range of the southern Udzungwa Mountains of Tanzania. Captive survival assurance populations of the spray toad have been established at a zoo in the United States. There are future plans to use offspring from the captive population to reintroduce the spray toad to Tanzania after mitigation of environmental factors in the native range.

Using this information in the decision tree:

- The spray toads are part of a captive breeding program that aims to reintroduce animals back into the wild. Therefore, these toads have an Ark, Rescue or Supplementation role.
- The breeding facility for the spray toads is considerably outside of the native range of this species. In this situation, the ISOLATION level of biosecurity is necessary to prevent the introduction of “non-native” amphibian pathogens to the spray toad populations. If “non-native” pathogens are introduced to the spray toads, there is a risk that these pathogens will also be introduced back to wild amphibian populations in Tanzania as part of the spray toad reintroduction effort.

4.5 SUMMARY OF BEST PRACTICES AND ISOLATION BIOSECURITY LEVELS

Best Practices

BEST PRACTICES are recommended for all captive amphibians, and specifically for those that:

- Are maintained in “long term isolation” because they have an ARK/RESCUE/SUPPLEMENTATION role, but are kept in a facility that is within the native range of the species or species assemblage and does not maintain or have contact with amphibians from outside the native range.

OR

- Have a CONSERVATION RESEARCH or CONSERVATION/EDUCATION role with no intention of returning the animals or their progeny to the wild. These animals can be within or outside of the native range. If these animals will be returned to the wild they should be considered to have an ARK/RESCUE/SUPPLEMENTATION role.

BEST PRACTICES will reduce the following types of risks of infectious disease in a collection:

- Acquisition of infectious diseases that:
 - Have a negative impact on the health of individual animals.
 - Interfere with the success of captive breeding or the sustainability of a captive amphibian program.

- Have a negative impact on the validity of any research that is conducted with a species.
- Inadvertent introduction of a non-native amphibian pathogen to a new location (e.g., frogs from the United States brought to a facility in Australia introduce a non-native *Ranavirus* to Australia).

Isolation

ISOLATION criteria are recommended for those animals that:

Have ARK/RESCUE/SUPPLEMENTATION role and they or their progeny are likely to be returned to the wild

AND

- Have been removed from their native range for *ex situ* conservation efforts (e.g., native to India and incorporated into *ex situ* conservation program in the Australia).

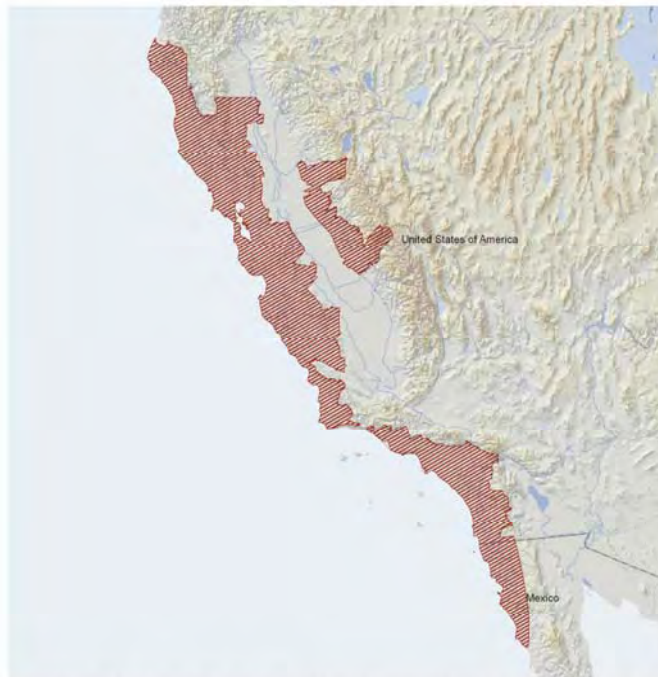
OR

- Are in *ex situ* conservation programs in the native range, but could be exposed to amphibians from outside the native range (e.g., a zoo in Germany that has an *ex situ* conservation program for native German species, but also keeps amphibians from other regions such as Kenya or the United States).

Mixing of individuals that come from allopatric populations of the same species may also require ISOLATION under some circumstances. For example, consider the distribution maps (below) for the hylid frog *Anothea spinosa* and the plethodontid salamander *Aneides lugubris*. These are examples of species with naturally occurring disjunct populations. In such cases, one must consider the concept of “inside” vs. “outside” the range of the species with special care, taking into account the separate populations that may have differing disease profiles in the wild. Similarly, in such cases, it may be advisable to maintain in isolation colonies deriving from different, disjunct, portions of the overall range of the species.



Distribution of the hylid frog *Anotheca spinosa* in Central America.



Distribution of the plethodontid salamander *Aneides lugubris* in western North America.

(source: www.iucnredlist.org)

ISOLATION will reduce the following types of risks of infectious disease in a collection:

- All risks covered by BEST PRACTICES.
- Additional safeguards to ensure that animals involved in ARK/RESCUE/SUPPLEMENTATION roles do not become infected with non-native

pathogens. The great risks presented by non-native amphibian pathogens are discussed in Section 4.3.

Change in Biosecurity Level

Animals must be maintained at the security level appropriate for their role. It is wise to maintain animals at the highest level of biosecurity (BEST PRACTICES or ISOLATION) necessary for the current as well as any future anticipated role(s) of the captive population.

- This is most important for animals that are kept in ISOLATION because they or their progeny will be returned to the wild. If these animals (or their progeny) are maintained with BEST PRACTICES, they are at higher risk for acquiring a non-native amphibian pathogen and introducing this pathogen into wild amphibian populations.
- If the role of the species changes (e.g., from ARK/RESCUE/SUPPLEMENTATION to CONSERVATION/RESEARCH or EDUCATION, the biosecurity level can be decreased (e.g., ISOLATION to BEST PRACTICES), but it is not appropriate to change the role of these animals back to the category of ARK/RESCUE/SUPPLEMENTATION role at a later time.

If animals previously maintained with BEST PRACTICES must be considered for an ARK/RESCUE/SUPPLEMENTATION role, the process of disease risk assessment is complicated, expensive and time-consuming and may not result in animals that are suitable for release into the wild (See Chapter 3).

4.6 BIOSECURITY PRACTICES

An outline of specific practices for the BEST PRACTICES and ISOLATION is given below in Table 4.1 and details of these practices are provided in subsequent sections. The major difference between the BEST PRACTICES and ISOLATION is the simple act of isolating the latter animals. The process of long-term isolation is described in detail in Section 4.8.

The specific husbandry practices described in subsequent sections accomplish one or more of the following goals:

- Prevent non-native amphibian pathogens from becoming introduced to new geographic locations and amphibian populations (“Nothing gets out”). This protects wild amphibian populations from new infectious disease risks.
- Prevent amphibian pathogens from being introduced into healthy captive amphibian populations (“Nothing gets in”). This is important for the sustainability of captive populations, the success of captive breeding and rescue programs and for maintaining animal welfare standards.
- Prevent amphibian pathogens from becoming transmitted between different animals within a captive amphibian facility (“Nothing gets around inside”). If infectious disease outbreaks occur within a facility, these husbandry practices can minimize the number of animals that will become affected.

Table 4.1—Outline of Husbandry Practices for BEST PRACTICES and ISOLATION.

Practice	Best Practices	Isolation
Long-term isolation of the species or species assemblage (Section 4.8)		+
Dedicated footwear for each long-term isolation room (Section 4.10)		+
Dedicated clothing for each long-term isolation room (Section 4.10)		+
Animals in long-term isolation cared for first in the day (Section 4.11)		+
Dedicated tools and equipment for each long-term isolation room (Section 4.13)		+
Dedicated footwear and clothing for each building (Section 4.10)	+	+
Wash hands or use disposable gloves between EACH enclosure (Section 4.10)	+	+
Follow a husbandry routine that reduces the potential for disease transmission (Section 4.11)	+	+
Clean and disinfect tools between different enclosures (Section 4.13)	+	+
Determine need for special wastewater treatment (Section 4.14)	+	+
Determine need for solid waste disposal (Section 4.15)	+	+
Disease free water and food sources (Sections 4.16 & 4.17)	+	+
Automation of husbandry practices if possible (Section 4.18)	+	+
Quarantine period for new animals entering a collection (Chapter 6)	+	+
Disease surveillance/necropsy of animals that die in collection (Chapter 9)	+	+

4.7 STAFF TRAINING AND IMPLEMENTATION OF BIOSECURITY PRACTICES

Animal husbandry staff members are one of the most important means (vectors) by which infectious diseases can be transmitted in an amphibian facility. Therefore, proper staff training and good standard operating procedures are the best methods for the control and prevention of infectious disease problems in captive amphibian conservation programs.

Suggestions that can help animal husbandry staff in carrying out biosecurity practices and that minimize the possibility of error include:

- Development and adherence of user-friendly written Standard Operating Procedures (SOPs) that provide an overview of biosecurity practices. These should be customized for each amphibian facility. The SOPs should be easily accessible to staff members. SOPs should be practical and developed with the consideration for the unique husbandry needs of each facility and the amphibian species being housed.
- Review biosecurity practices with new staff members before they begin working with animals. Annual review of biosecurity practices with all animal care staff is also suggested.
- Advanced training in biosecurity measures and good amphibian husbandry practices can be promoted by specialist educational programs for amphibian keepers. For example, the American Association of Zoos and Aquariums has a model course in Amphibian Biology and Management with the goal to “provide a solid background in amphibian biology as it relates to husbandry, breeding, conservation and cooperative programs” (<http://www.aza.org/prodev/Amphibians/>).
- Provide husbandry staff with the tools and equipment necessary to manage captive amphibian populations at appropriate biosecurity levels for the role of the species that they care for.
- Errors in biosecurity practices will occur. Encourage a work environment where staff members feel comfortable reporting these errors as soon as they occur. If errors are identified early more can be done to minimize their impact.
- When formulating SOPs design procedures that reduce the amount of staff contact with amphibians (automation). These procedures should still allow staff to regularly observe subtle signs of disease or abnormal behavior. Staff should assume that all enclosures have the potential to contain animals with infectious diseases and follow routines that minimize the possibility of disease transmission (see Section 4.11).

4.8 LONG-TERM ISOLATION OF AMPHIBIANS DESTINED FOR REINTRODUCTION TO THE WILD

Amphibians kept in conservation programs that have a goal of reintroducing captive animals or their progeny to the wild should be permanently separated and protected from other amphibians in a cosmopolitan zoo or other amphibian collections in “long term isolation”. Programs that have been established within the range country of the species or species assemblages maintained in the facility and that do not maintain

any amphibians from outside the range country are the best and most effective examples of long-term isolation.

- Long-term isolation has also been called “permanent quarantine” by the 2006 CBSG/WAZA Amphibian *Ex Situ* Conservation Planning Workshop (Zippel et al., 2006) or “Quarantine 1” and “Quarantine 2” by the 2008 Association of Zoo and Aquariums Amphibian Husbandry Resource Guide (Kast and Hanna, 2008).
- The term “long-term isolation” is used in this manual to distinguish “permanent quarantine” from the quarantine used when adding new animals to an amphibian collection (see Chapter 6).

Long-term isolation is accomplished by:

- Housing only a single species or species assemblage (an amphibian faunal group that naturally occurs together in the range country) in a freestanding building, or inside of an isolated room or rooms within a building. Details for creating facilities for long term isolation are discussed below.
- The greater the physical isolation of a species or species assemblage from a cosmopolitan amphibian collection the simpler it is to establish and maintain long-term isolation and effective biosecurity practices (e.g., a separate building for long-term isolation is better than separate rooms within a building).

↑ separation of isolated populations = ↓ difficulty of maintaining biosecurity

- Animals in long-term isolation are never housed in the same room with amphibians from outside their native range (e.g., A Mississippi gopher frog from the United States should not be kept in the same room as a Kihansi spray toad from Tanzania).
- Preventing indirect contact with amphibians from outside the native range by potential vectors including animal care staff, cages, substrate, water systems, or tools. In other words, nothing that has come into contact with amphibians from outside the native range should come into contact with animals held in long-term isolation. This involves implementation of specific biosecurity practices that are discussed in detail in Sections 4.10, 4.11 and 4.13 below.

4.9 FACILITIES FOR LONG-TERM ISOLATION

Isolated rooms within a building

The development of dedicated rooms for long-term isolation within a cosmopolitan amphibian facility is a cost-effective method for achieving ISOLATION for captive amphibian populations. This approach may be cost effective, but it is not ideal, because of the increased likelihood that cross-contamination may occur during times of water leaks, flooding, or even simple inadvertent violation of quarantine.

- If dedicated rooms are used the potential for a significant error in facility biosecurity is greatly increased. Animal husbandry staff must be committed to the concept and practice of ISOLATION guidelines for staff work-flow, clothing and footwear, and use of tools and equipment.

The rooms used for long-term isolation can be converted from rooms that already exist within the amphibian facility or can be specially constructed. Special and relatively inexpensive (approximately \$ 7000 US) construction of modular long-term isolation rooms in otherwise unused space within a zoo has been described in detail from Omaha's Henry Doorly Zoo (Krebs, 2008; See Appendix 3). Long-term isolation rooms should be considered individual units with modifications that prevent the entry or exit of amphibian pathogens.

Specifications for rooms that reduce disease transmission risks include:

- Rooms should be sealed to prevent escape of water or amphibian waste into adjacent rooms (Section 4.18).
- Rooms should be escape-proof and pest-proof (see Section 4.18).
- Rooms and surrounding corridors should be designed to easily qualify for ISOLATION, with regards to staff work-flow, clothing and footwear (Section 4.18).
- If modular rooms are constructed plans should be made for regular maintenance to prevent breakdown of construction materials (e.g., sealants used between walls and floor as water barrier).
- Consideration should be made of air handling and movement within the facility (Section 4.18).

Separate Long-Term Isolation Buildings

As noted earlier, development and construction of separate long-term isolation buildings further reduces disease risk. A successful approach developed by the Australian Amphibian Research Center for creating inexpensive long-term isolation buildings uses modified large cargo shipping containers. Details on construction of these containers can be found online (<http://frogs.org.au/arc/container.php>). Shipping containers range from 6–12m in length and are outfitted with independent air cooling/heating, water and electrical systems and designed to utilize husbandry space in an efficient manner. It is possible to retrofit containers prior to installation on-site, and therefore they can be installed with less construction support compared to new building construction. These features make shipping containers a flexible and economical option for many amphibian programs. The units can also be built with viewing windows for exhibition purposes in order to increase public support, funding opportunities, and educational opportunities for the general public.

4.10 HUSBANDRY STAFF HYGIENE AND PROTECTIVE CLOTHING

Procedures for amphibian facility husbandry staff hygiene and protective clothing are important for the success of biosecurity protocols. This section makes recommendations for amphibians maintained either with BEST PRACTICES or in

ISOLATION. Invitation of visitors to biosecure areas should, in general, be discouraged. When necessary, visitors are to follow the same guidelines as regular staff members.

Footwear and Disinfectant Foot Baths

- BEST PRACTICES dictates that dedicated footwear should be required for each building that houses captive amphibians. It is important that husbandry staff or visitors not enter animal care areas with footwear that has been used outside of the captive facility (e.g., at their homes or in the field).
- In ISOLATION, dedicated footwear is required for each long-term isolation room. Dedicated shoes or boots can be stored within the isolation room. The goal is to avoid tracking organic material or amphibian pathogens from one long-term isolation room (that contains a different species or species assemblage) to another, or from areas of BEST PRACTICES into areas of ISOLATION. An alternative to dedicated footwear is the use of disposable plastic foot covers (e.g., Shubees™).
- Disinfectant foot baths are used in some animal facilities to clean footwear between animal rooms. Foot baths are only effective if footwear is made of easily disinfected material (e.g., rubber boots) and is not heavily contaminated with soil or other organic material. Reviews of the use of disinfectant foot baths are available (Morley et al., 2005; Dunowska et al., 2006).
 - Foot baths require a high degree of maintenance to avoid the build up of organic materials that inactivate disinfectants and to avoid evaporation of the disinfectant solution.
 - Footbaths might be useful when placed at the entrance and exit of biosecure areas to remove primary matter, and to remind staff that they are entering a biosecure area and should remain mindful of biosecurity protocols.
 - Disinfectants for use in foot baths include sodium hypochlorite (bleach), Virkon, and F10. Virkon may have advantages for use in footbaths because it maintains greater activity in the presence of organic materials. (See Chapter 5).
 - For most situations use of dedicated footwear rather than footbaths is preferable.

Dedicated Clothing

- For BEST PRACTICES dedicated clothing is required for each building that houses captive amphibians. It is important that husbandry staff or visitors not enter animal care areas with footwear that has been used outside of the captive facility (e.g., at their homes or in the field). Dedicated facility uniforms that are regularly laundered are sufficient. Uniforms should be changed if they become wet or heavily contaminated with organic material.
- For ISOLATION dedicated protective clothing is required for each long-term isolation room. In most circumstances it will be sufficient to have a separate laboratory coat or other coverall that is placed over the staff member's regular

clothing or uniform. If the regular clothing is wet, dirty or otherwise contaminated with material from outside the long-term isolation room, a full-change of clothing is required before entering a long-term isolation room. Alternatives include the use of disposable protective clothing for each long-term isolation room (e.g., Tyvek[®] jumpsuits or surgical “scrubs”).

Hand-Washing and Use of Protective Gloves

- 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 in programs operating in both BEST PRACTICES and ISOLATION. It is especially important to wash the hands and arms:
 - Before entering each ISOLATION rooms or facilities.
 - In-between working on different enclosures as standard part of both BEST PRACTICES and ISOLATION protocols.
- Disposable gloves should be used when handling amphibians or cleaning enclosures.
 - A new pair of gloves should be worn for each enclosure.
 - Non-powdered gloves should be used or gloves should be thoroughly rinsed before handling animals.
 - A recent study (Cashins et al. 2008) suggested that latex and nitrile gloves might be toxic to some tadpoles. Vinyl gloves were also shown to be toxic if they were not rinsed with water prior to exposure. The observation of toxicity associated with glove use has not been consistent and recommendations for addressing this issue have been published (Greer et al., 2009).
 - Toxicity associated with glove use has **not** been observed in postmetamorphic animals.
 - One experimental study shows increased survival times for the amphibian chytrid fungus on bare hands that had been repeatedly washed with ethanol and water (Mendez et al., 2008). The implications for disease transmission in a natural setting are unclear and hand-washing was still found to be preferable to continuous re-use of disposable gloves.

4.11 HUSBANDRY ROUTINES

The husbandry routines and work-flow pattern used by amphibian husbandry staff are important for minimizing the potential to move pathogens within a captive amphibian facility including:

- Transmission of pathogens between animals kept at different levels of biosecurity.
- Transmission of pathogens between amphibian enclosures.
- Transmission of pathogens between animals in quarantine (see Chapter 6) and animals in an established amphibian collection or animals held in the ISOLATION level of biosecurity.

Animals that are kept in ISOLATION should be cared for first in the day before taking care of animals maintained by BEST PRACTICES. This is also true when animals intended for ISOLATION are brought into quarantine (see Chapter 6). Animals in ISOLATION should never be housed in the same quarantine room as animals from outside their original host range.

Automation of amphibian husbandry tasks such as feeding and cleaning can reduce direct contact time with amphibians and minimizes possibilities for disease transmission. Suggestions for automation are provided in Section 4.18.

A systematic organized routine for the daily care of a collection is highly recommended. The routine must be specific so that if a problem does occur, each step can be reproduced eliminating areas of risk, and determining where exactly the error occurred or pathogen was introduced. 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. A concise list stating the direction in which to proceed with routine husbandry practices would be preferred and should be clearly stated and posted for all who maintain specimens to see and understand. For example, tanks could be labeled sequentially, and the procedure could dictate that one proceeds from:

Enclosure A → Enclosure B → Enclosure C

- Enclosures that contain amphibians that are least likely to be infected with pathogens of concern (e.g., animals that have been in captivity for a long time or animals that have tested negative for *Bd* or other diseases of concern) should be arranged so that they are serviced first in the directional sequence (e.g., Tanks A–D, of 15 total tanks).
- Enclosures and equipment associated with the enclosures (e.g., lighting and filtration units) should be labeled to clearly identify each unit in the sequence of enclosures.
- If sick or dead animals are found during the husbandry routine they should immediately be removed from the enclosure. Dead animals are submitted for necropsy examination (see Chapter 9). Sick animals are removed for veterinary attention. At a minimum, staff members should wash their hands before returning to complete the directional servicing in that room or facility.
- Incorporate these procedures into the facility Standard Operating Procedures (See Section 4.7) and provide standardized training for daily husbandry staff in their implementation.

4.12 ENCLOSURE SANITATION

Regular cleaning of amphibian enclosures is essential at all levels of biosecurity to reduce build-up of organic materials that may increase the risk of infectious and parasitic diseases in captive amphibian populations. Some general concepts of enclosure sanitation that are important from a biosecurity standpoint include:

- The frequency with which amphibian enclosures should be cleaned increases as amphibian biomass (number of animals) and feedings increase.

- Frequency of water changes is dependent on many factors including availability and use of filtration systems.
- Tanks must 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. Recommendations for disinfectants to use on tanks and equipment are provided in Chapter 5.
- Equipment such as racks, shelves, counters, and also floors should be constructed of materials that are easy to wash/mop, disinfect, and rinse. A regular cleaning and disinfecting schedule of all exposed surfaces is necessary.
- For animals kept in ISOLATION, it is important to wash equipment and enclosures only in sinks or areas that have been carefully disinfected and are free of contaminated materials from other animals in the amphibian collection. This includes materials from other animals held in ISOLATION biosecurity that are from a different geographic region (e.g., tanks used for animals in ISOLATION from Panama should not be cleaned in the same sink as those from animals in ISOLATION from the United States without complete disinfection). Alternatively, ISOLATION rooms can be constructed with dedicated sinks for each room.

4.13 EQUIPMENT, ENCLOSURE SUBSTRATES, AND CAGE DECORATION

The tools, equipment and enclosure substrates used in an amphibian facility can be important vectors for the introduction of new pathogens to a captive population or for the transmission of pathogens between enclosures and different groups of animals.

Recommendations for reducing these risks include:

- ISOLATION rooms should have a dedicated set of equipment (e.g., nets, forceps, suction tubing, scrub brushes, sponges etc.). This equipment does not leave the individual isolation room and is never used on a different group of animals.
- For both the BEST PRACTICES and ISOLATION protocols the husbandry staff should assume that all amphibians and amphibian enclosures are a potential source of pathogens that can be transmitted to another enclosure. This is regardless of the presumed health of the animals. For example, a frog might appear to be very healthy, but can still act as a carrier of the chytrid fungus.
 - Tools and equipment are cleaned and disinfected between use in different enclosures (e.g., a net used in Enclosure A is disinfected before use in Enclosure B); alternatively, nets or other minor tools may be dedicated per enclosure.
 - Multiple sets of equipment may be necessary (one set of equipment can be disinfected while the other set is in use). Tools are labelled to corresponding tanks or rooms for easy recognition.
 - Guidelines for disinfection of tools and equipment are provided in Section 5.5). Care should be taken not to disinfect or clean enclosures or equipment from one group of animals in the same sink or area as equipment from a different group of animals without first cleaning and disinfecting the sink or cleaning area.

- Enclosure substrates, cage furniture and decorations (e.g., plants and rocks) of any kind are not moved from one enclosure to a different enclosure without disinfection. Some enclosure substrates such as sphagnum moss or soil should be considered disposable and not moved between enclosures. Porous materials such as wood or cork are difficult to disinfect. Guidelines for disinfection of substrates and plants are provided in Section 5.6–5.7.
- Whenever possible tools, equipment and cage furniture should be made of materials that are easily cleaned and disinfected (e.g., plastic, metal, glass).
- Use of natural materials (e.g., soil, gravel, rocks, plants) in amphibian enclosures can be important for supporting normal behaviors (including breeding), reducing stress and for decoration of display enclosures in zoos.
 - Natural materials do have the potential to be contaminated with amphibian pathogens and the source of these materials as well as disinfection should be carefully considered before placement into an enclosure.
 - When selecting natural materials for use in enclosures the likelihood that there has been exposure to native or non-native amphibian fauna, insecticides and fertilizers should be considered.
 - Materials from areas with known amphibian disease problems should be avoided (e.g., plants from the site of an ongoing outbreak of chytridiomycosis). Plants that have been grown hydroponically (in water) or in a dedicated greenhouse (without native amphibian colonization) may reduce disease risks. The use of artificial (plastic or silk) plants can also be considered especially for use in the animals in ISOLATION.
 - Suggestions for disinfection or sterilization of natural materials are found in Section 5.6

4.14 WASTEWATER DISPOSAL

Disposal of wastewater is an important biosecurity consideration in the development and management of captive amphibian facilities. Best practices require that pathogens not be discharged into the environment. The most important biosecurity concern for wastewater disposal is the potential to introduce non-native amphibian pathogens to new geographic locations by discharge of untreated wastewater to the environment.

- For facilities that keep amphibians from outside the native range (e.g., Panamanian amphibians kept in the United States) or that conduct research with amphibian pathogens wastewater biosecurity measures are needed (details below). This applies to animals under either with BEST PRACTICES or in ISOLATION. It is not acceptable to release wastewater to the local environment.
- A facility that only keeps amphibians from inside the native range (e.g., southern Japanese amphibians kept in southern Japan) usually does not need to consider wastewater biosecurity measures.

Implementation of water biosecurity measures can be complicated and expensive and is a major reason why keeping captive amphibians within the natural range country of the

species is strongly preferable. Unfortunately, amphibian wastewater biosecurity is a controversial topic because much of the information needed for evidence-based decision making is simply not available. Organization of a working group to perform a risk-assessment of amphibian wastewater management is sorely needed. This working group should include experts in wastewater treatment (especially experts from intensive aquaculture), sanitary sewer systems, environmental microbiology, amphibian disease, and amphibian facility design/husbandry.

Factors that must be considered for wastewater biosecurity include:

- The amount of water that must be treated (e.g., a small captive breeding facility can accommodate different water treatment methods than a large commercial facility that raises frogs for food).
- The infrastructure of general wastewater (sewage) treatment for a geographic region or country.
- Financial and other resources available.

There are a variety of wastewater treatment and disinfection options available for installation in a captive amphibian facility. Each option has its own advantages, disadvantages, and resource requirements. No water treatment option is absolutely failsafe for removal of potential amphibian pathogens and options are especially limited when large quantities of water effluent must be treated.

Options for wastewater treatment include:

- **Modern municipal wastewater (sanitary sewer) system.** Modern sanitary sewage treatment systems are adequate for disposal of wastewater from many small to medium sized amphibian facilities without additional disinfection and at low risk to wild amphibian populations. For instance, in many areas it is considered acceptable to directly release liquid infectious waste from humans and domestic animals into the sanitary sewer. However, the sanitary sewer option has limitations that need to be seriously considered:
 - It is not acceptable to discharge large amounts of waste or large quantities of infectious agents into the sanitary sewer without disinfection. Very large amphibian facilities (e.g., frog farms), facilities experiencing outbreaks of infectious disease, facilities that regularly import large numbers of frogs from outside the range country and facilities conducting infectious disease research should take additional measures to disinfect water before discharge into a sanitary sewer.
 - It should not be automatically assumed that local sanitary sewer systems are adequate for biosecurity. Local systems should be individually evaluated for effectiveness and reliability. For instance, the actual treatment facility may be adequate, but the plumbing (sewers) that feed the system could be prone to failure and discharge of infectious material into the local environment. In other cases, the local storm drains are connected to the sewage drains and when it rains, the treatment facilities

are overwhelmed from storm water and discharge untreated sewage into the environment.

- **Processes for Disinfection or Sterilization of Wastewater.** There are a variety of options for treatment of wastewater to inactivate infectious agents prior to disposal either in a sanitary sewer or to the environment include application of chemicals (sodium hypochlorite “bleach”), physical agents (heat and pressure), ozonation, and ultraviolet radiation. Details and limitations of some of these methods are provided in Section 5.8 and in Appendix 2.
 - Automation of the water disinfection methods in a facility is suggested. Automation minimizes human error; ensures that disinfectant concentrations are appropriate; ensures that contact time with disinfectant is adequate; and makes wastewater treatment simple for animal staff to implement. An automated wastewater disinfection system (AWWDS) for amphibian captive breeding facilities (using sodium hypochlorite) has been described (Robertson et al., 2008). Manual methods for water disinfection using sodium hypochlorite have also been described (Krebs, 2008).
 - Water disinfection systems and protocols for use in large-scale commercial aquaculture are a valuable source of information for system design and implementation. Many of these systems are based on ozonation of wastewater (Schuur, 2003). Large scale amphibian breeding facilities or farms should follow the guidelines of the World Organization for Animal Health (OIE) for disinfection of effluent wastewater in aquaculture facilities (www.oie.int/eng/normes/fmanual/1.1.3_DISINFECTION.pdf).
 - Water disinfection systems should be designed to minimize introduction of toxic chemical contaminants (such as 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 UV radiation (Robertson et al., 2008).

4.15 SOLID WASTE DISPOSAL

The most important biosecurity goal of procedures for solid waste disposal in captive amphibian facilities is the same as for wastewater disposal—to prevent the introduction of non-native amphibian pathogens to new geographic locations. Therefore considerations for solid waste disposal are most important for facilities that keep amphibians from outside of the native range or that conduct research with amphibian pathogens. Solid wastes from these facilities should not be discharged into local environment without treatment.

Options for disinfection or sterilization of solid wastes prior to disposal include:

- Autoclaving.
- Incineration (not environmentally friendly).
- Alkaline hydrolysis (amphibian tissues or carcasses).

Deep burial or transfers to a modern landfill are also acceptable options for waste disposal without the need for disinfection. The key is to contain infectious material and prevent native amphibians from becoming exposed to this material.

4.16 SOURCES OF WATER

The source and composition of water for an amphibian facility is a critically important consideration for the success of any captive amphibian conservation program. Details on water sources, quality and treatment for amphibian facilities have been reviewed in a recent publication (Browne et al., 2007). Items that must be addressed include pH, water hardness, trace elements, presence of potential toxic metals (e.g., copper) and the presence of potentially toxic additives (e.g., chlorine or chloramines in municipal water supplies or environmental contaminants such as pesticides).

- It is important that water used in a captive amphibian facility be free of important amphibian pathogens. This is true for both BEST PRACTICES and ISOLATION programs. If modern municipal water supplies are used as an initial source for facility water, the risk of disease introduction is very low. If natural water sources are used, consideration should be given to disinfection of incoming water prior to use in amphibian enclosures. Potential methods of disinfection include application of physical methods (e.g., heat and pressure) and filtration (see Section 5.8).

4.17 SOURCES OF FOOD

Food items offered to amphibians in captive facilities are a potential source for introduction of pathogens to a population. This is a concern for animals maintained both with BEST PRACTICES or in ISOLATION. 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.

- When possible invertebrate food items should be cultured on-site at the amphibian facility. Aquatic food items (e.g., *Daphnia* or brine shrimp [*Artemia*]) should be started from eggs if possible.
- If food items are purchased from a commercial supplier, the amphibian facility should make sure that practices that reduce disease risk are used. Ideally, food items should be reared indoors without the potential for exposure to the elements, amphibians of any kind (wild or captive) or to wild insects. The supplier's facility should be clean and use high-quality materials.
- There are potential benefits to feeding wild-caught food items to captive amphibians (e.g., superior nutrition or the amphibian species has unique dietary requirements and preferences). These benefits must be considered with the disease risks when making decisions for a captive population. Animals kept in

ISOLATION should not be fed wild-caught food items from outside of their native range (e.g., Panamanian frogs kept in a survival assurance population in the United States should not be fed wild-caught insects from the United States).

- If wild-caught food items are fed to captive amphibians, these food items should only be collected from areas that are known to be free of pesticides or other chemicals. Some pesticides can persist in the environment for several years, so it is recommended as a general (and admittedly arbitrary rule) that food insects be collected at least 0.5 km away from any areas where pesticides have been applied in the past three years.
- Wild-caught food items should not be collected from locations known to be experiencing outbreaks of amphibian infectious diseases (e.g., do not collect aquatic invertebrates from a stream where amphibians are dying of chytridiomycosis).

4.18 FACILITY DESIGN AND BUILDING SPECIFICATIONS

Amphibians have special requirements for water systems and filtration, climate control and light that are essential for overall animal health and success of breeding colonies in captivity. Complete overviews of amphibian facility design and husbandry standards have been recently published and these resources should be consulted when designing or renovating a facility (Browne et al. 2007; Pramuk and Gagliardo, 2008).

Elements of facility design that are important for biosecurity are highlighted below:

- **Facilities should be pest-proof.** Free-ranging pests (e.g., cockroaches, ants, or rodents) can occur in any captive amphibian facility. Dangers of pests include direct injury or killing of amphibians; destruction of enclosures which allow for amphibian escape; and transmission of amphibian pathogens (either between amphibians within the facility or to wild amphibians outside of the facility). Facility design features to minimize risks from pests include: tight-fitting gaskets on doors and windows; self-closing doors to amphibian rooms; screened floor drains; and addition of baffles to air conditioning units. Physical pest control methods can be used within the facility (e.g., insect traps and fly paper). Chemical pest control methods should be avoided because of the risk of poisoning the facility amphibians.
- **Facilities should be amphibian-proof.** Facilities and enclosures should be designed to prevent the escape of captive amphibians and prevent the entry of wild amphibians from outside the facility. This is most important for amphibians kept in ISOLATION.. The risk in this situation is that non-native amphibians could escape from the facility and introduce non-native amphibian pathogens to native wild amphibians or that native amphibians could introduce new pathogens to non-native amphibians in the facility. BEST PRACTICES dictate that native amphibians could still introduce a pathogen that has been controlled or eliminated in the captive population (e.g., the captive population has been treated and cleared of infection with the chytrid fungus, but local wild amphibians could serve as source of re-infection of the captive animals). An example of amphibian-proofing is to add screen coverings to drains in amphibian rooms.

- **Husbandry routines should be automated.** Using automated systems for draining enclosures, adding water to enclosures, filtration and feeding animals are encouraged to minimize keeper/animal contact and reduce probability of human errors. Automation is an important measure that can reduce potential for disease transmission between individual enclosures. For feeding, a funnel can be placed through each enclosure lid and secured to allow for the feeding of prey items into the enclosure without the need for the keeper to open, or otherwise contact the enclosures. The funnel neck should be small enough and placed appropriately so that it does not allow the amphibians to escape; the funnel can be capped after prey items are introduced to prevent their escape as well. Details on facility automation can be found at the Amphibian Research Center website ([/frogs.org.au/arc/features.php](http://frogs.org.au/arc/features.php)).
- **Air distribution systems.** Airborne transmission of significant amphibian pathogens has not been documented to date. Although the focus of facility biosecurity efforts should be on movement and introduction of pathogens by contact with husbandry staff, other amphibians and substrates and tools, the potential for airborne transmission should not be entirely ignored. For facility design, air distribution systems that prevent air from being forced between rooms are desirable. Shared ductwork between different rooms that hold animals at the ISOLATION should be avoided.
- **Water proofing and sanitation.** Amphibian facilities are wet and humid environments, therefore walls, ceilings, and floors must be waterproof—both to maintain the integrity of the room or building, and to facilitate regular cleaning and disinfection of those surfaces. In addition, seals help to prevent leakage of amphibian wastes from areas containing cosmopolitan collection animals or other amphibians from different geographic regions into rooms used for animals held at ADVANCED biosecurity levels. Drywall (plasterboard) and cellulose ceiling materials are difficult to disinfect and are not recommended.
- **Design of facilities to accommodate the needs of ISOLATION.** Rooms that hold animals held in ISOLATION are greatly improved by design features such as anterooms that allow for easy change of clothing and footwear prior to entry and by placement of sinks in the individual rooms to eliminate the risk of using communal sinks (used to clean tools and materials from cosmopolitan collection animals or different groups of animal held in ISOLATION).

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Chapter 5

CLEANING AND DISINFECTION

5.0 INTRODUCTION

The ability to thoroughly clean and disinfect a huge variety of equipment, animal enclosures, cage decorations and furniture and even water is essential for good biosecurity and control of infectious diseases in captive and wild amphibian populations. This chapter discusses cleaning and disinfection with an emphasis on the amphibian chytrid fungus and ranaviruses, however, the concepts are applicable to a wide variety of amphibian pathogens.

Concepts emphasized at the workshop included:

- Disinfectants are inactivated by the presence of dirt or organic materials. Therefore thorough cleaning of objects prior to disinfection is absolutely essential.
- A single method or type of disinfection will not work for all amphibian pathogens. Careful selection of the disinfectant type is necessary for different situations.
- Disinfectant concentrations and contact time with disinfectants are important for effective disinfection.
- Environmental impacts should be considered when selecting a disinfectant (if possible). Quaternary ammonium compounds and potassium peroxymonosulfate (Virkon®) have advantages in this regard over sodium hypochlorite (bleach).
- Glass or metal surfaces and materials are easy to properly disinfect with chemical disinfectants.
- Plastics, of varying types (including silicon), are easy to disinfect, but may absorb chemicals from the disinfectant (e.g., scenting chemicals, solvents) thus rendering the enclosure itself to be toxic. This likely is an under-appreciated problem.
- Natural materials such as rocks, wood or dehydrated plant materials are difficult to disinfect. Application of heat may be most effective.
- Living plants are also difficult to disinfect. Careful plant selection, removal of dirt and gentle chemical or physical disinfection may reduce risks.
- Methods for water disinfection include heat, filtration, chemical disinfection or ozonation.

Of the disinfectants available:

- Sodium hypochlorite (bleach), quaternary ammonium compounds and potassium peroxymonosulfate (Virkon®) are experimentally effective against the amphibian chytrid fungus. However, sodium hypochlorite must be used in high concentrations. The quaternary ammonium compounds have an advantage because they are effective in low concentrations. The chytrid fungus also is susceptible to relatively low levels of heat and to complete drying (desiccation).
- Potassium peroxymonosulfate (Virkon®), sodium hypochlorite and chlorhexidine are experimentally effective against ranaviruses. However, experiments have only been shown effective on wet materials or cell cultures. These viruses may be somewhat resistant to disinfection on dry surfaces (studies are limited).

5.1 PRINCIPLES OF CLEANING & DISINFECTION

Excellent principles of cleaning and disinfection are vital to the control of infectious diseases in captive amphibian populations and to successful implementation of biosecurity practices. Designing an appropriate disinfection protocol requires understanding of the properties of disinfectants and target pathogens, and practical consideration of the equipment, facilities or processes requiring disinfection. As well as understanding the efficacy of various disinfecting processes, it is important to consider the safety of any disinfection protocol to the environment and the amphibians.

There are a few useful definitions for terms used in this chapter:

- **Cleaning:** The physical removal of all visible organic and inorganic debris from items such as dirt or fecal material (Kast and Hanna, 2008).
- **Disinfection:** A physical (e.g., UV light) or chemical (e.g., chlorine bleach) process to reduce the numbers and viability of microorganisms (e.g., bacteria, fungi, or viruses) on an object, surface or material.
- **Sterilization:** A physical or chemical process that removes all microorganisms from an object, surface, or material.

Thorough cleaning and disinfection reduce most of the risk of transferring amphibian pathogens. Sterilization of objects is labor intensive and less practical for most routine applications.

Guidelines available for cleaning and disinfection of commercial aquaculture facilities may be applicable to amphibian facilities, especially for amphibian farming and mass production facilities. The World Organization for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals has a succinct overview of aquaculture disinfection methods: www.oie.int/eng/normes/fmanual/1.1.3_DISINFECTION.pdf

Cleaning

The act of cleaning itself does not render an object free of pathogens that can cause amphibian diseases, however, it is an extremely important to thoroughly clean objects prior to disinfection or sterilization.

- Thorough cleaning physically removes many or most pathogens that are trapped in organic debris. By reducing the numbers of pathogens on an object the process of disinfection is more likely to be successful. Cleaning also allows disinfectants to directly contact the surfaces of an object.
- Many disinfectants are inactivated by the presence of organic material. Therefore in order for the process of disinfection to be successful an object must first be thoroughly cleaned.
- Warm or hot water improves the ability to remove organic materials from objects.
- General cleaning of all items involved in amphibian husbandry should be performed regularly. Cleaning is aided by use of detergents that loosen organic material from the surface of objects and help to break apart biofilms of microorganisms that can resist disinfection. Thorough rinsing of detergents from objects is essential after cleaning.

Disinfection

After cleaning, disinfection of an item by application of an appropriate chemical agent reduces the pathogen numbers and viability to a point where they are unlikely to serve as a source of infection. The choice of a disinfectant to use in an amphibian facility depends on:

- **Efficacy of the disinfectant and the type of pathogens that must be eliminated.** For example, some microorganisms such as *Mycobacterium* spp. or *Cryptosporidium* spp. are very resistant to most common disinfectants.
- **The potential for toxicity to amphibians that are exposed to the disinfectant.** Amphibians are very sensitive to disinfectant residues and thorough rinsing of all disinfectants is required after use.
- **Concerns about human exposure to disinfectants and about discharge of disinfectants into the environment.**
- **Safety for use on different materials.** Some disinfectants may be corrosive to materials or tools used in amphibian facilities
- **Ease of use and disposal.**
- **Cost.**

5.2 CHEMICAL DISINFECTANTS

There are a wide variety of chemical disinfectants available for use in captive amphibian facilities and in the field. Each disinfectant has unique advantages and disadvantages. Many disinfectants are inactivated by the presence of organic materials and thorough cleaning of surfaces is necessary prior to use. It has been suggested that disinfectants be used at concentrations greater than those determined to be effective against specific amphibian pathogens in the laboratory, in part, to overcome problems associated with residual organic material (especially in field situations) (Webb et al., 2007).

Disinfectant concentration and contact time are critical to the efficacy of a chemical disinfection regime. The recommended contact times for various disinfectants varies with the concentration used, and with specific pathogens that must be eliminated. For example, a 1% sodium hypochlorite solution will kill the amphibian chytrid fungus within 1 minute of contact time, whereas a 0.4% sodium hypochlorite solution requires a minimum of 10 minutes of contact time (Johnson et al., 2003). Recommendations and in-vitro effectiveness of various disinfectants are summarized in a table at the end of this chapter.

Chlorine (“Bleach”)

Chlorine, most often in the hypochlorite form, is the active ingredient in household bleach. Chlorine is an effective disinfectant against bacteria, fungi (including the amphibian chytrid fungus) and many viruses (including ranaviruses). Thoroughly cleaned surfaces and warm water enhance the disinfectant properties.

Advantages of bleach as a disinfectant are:

- Widely available.
- Low cost.
- Active against the amphibian chytrid fungus and ranaviruses at relatively high concentrations (Langdon, 1989; Johnson et al., 2003; Bryan et al., 2009).

Disadvantages of bleach as a disinfectant are:

- Easily inactivated by organic materials (i.e., dirt).
- Corrosive to metals, fabrics and silicone sealants especially at higher concentrations.
- Concerns that bleach may result in damage to aquatic environments (Webb et al., 2007; Schmidt et al., 2009).
- Chlorine is highly irritating to amphibians and other aquatic organisms, and at high levels can lead to toxicosis and death (Wright and Whitaker, 2001). Thorough rinsing of objects is required after disinfection. Avoid exposing animals to chlorine fumes during disinfection.
- May not be effective on ranaviruses on dry surfaces (Langdon, 1989).

Important note: The concentration of sodium hypochlorite in household bleach varies between commercially available products (between 4–6% sodium hypochlorite in most products). In addition, concentrated sodium or calcium hypochlorite products (up to 12% hypochlorite) are available for use in swimming pools or other large water volumes. When determining the amount of bleach to use to achieve appropriate disinfection, it is important to determine whether the recommended concentrations in a publication refer to the bleach product, the sodium hypochlorite content of the product, or the active ingredient (chlorine).

- Most publications that describe disinfection recommendations for amphibian pathogens refer to the concentration of sodium hypochlorite.
- For example, a sodium hypochlorite concentration of 1% has been shown to kill the amphibian chytrid fungus with a minimum contact time of 1 minute (Johnson et al., 2003). Using a household bleach product containing 4% sodium hypochlorite, a 1% solution of sodium hypochlorite is achieved using a dilution of 1 part household bleach to 3 parts water.
- In contrast, a 1% solution of the same household bleach product is achieved by diluting 1 part household bleach to 99 parts water which results in a sodium hypochlorite concentration of just 0.04% and this concentration is ineffective in killing the chytrid fungus.

Quaternary Ammonium Compounds (QACs)

The QACs are a large group of disinfectants commonly used in animal management that have antibacterial, antifungal and antiviral properties. Examples include benzalkonium chloride and didecyl dimethyl ammonium chloride (DDAC).

Advantages of QACs include:

- Less irritating and relatively non-toxic compared to chlorine.
- Biodegradable and fewer concerns about environmental damage in low concentrations.
- Not corrosive to metals, fabrics and other materials used in amphibian enclosures.
- Effective against the amphibian chytrid fungus in low concentrations. This is useful for field work since it allows small volumes of stock solution to be easily transported to remote locations (Johnson et al., 2003; Webb et al., 2007).
- Inexpensive and good for general facility and surface cleaning.

Disadvantages of QACs include:

- May become inactivated by soap residues or by organic materials (i.e., dirt).
- Efficacy of QACs against ranaviruses has not been determined (although they are effective against other large DNA viruses such as herpesviruses).

Examples of QAC products include:

- Path-X[®]
- QAC 128[®]
- F10 Super Concentrate Disinfectant[®]
- TriGene[®]
- Roccal[®]

Potassium peroxymonosulfate (Virkon[®])

Virkon S[®] and Virkon Aquatic[®] (Antec International/DuPont) are broad spectrum disinfectants active against many bacteria, viruses and fungi.

Advantages of Virkon[®] are:

- Effective against both the amphibian chytrid fungus and ranaviruses (Johnson et al., 2003; Bryan et al., 2009).
- Fewer environmental or amphibian toxicity concerns (Schmidt et al., 2009). Although amphibian safety requires additional investigation (Bryan et al., 2009).
- Not corrosive to materials or irritating to human skin at recommended concentration of 1%.
- Maintains more activity in the presence of organic materials than bleach.

Chlorhexidine (Nolvasan[®])

Nolvasan[®] (Fort Dodge Animal Health) is a common disinfectant used in veterinary hospitals and has antibacterial, antifungal and antiviral activity.

Advantage of Nolvasan[®] is:

- Activity against ranaviruses (Bryan et al., 2009).

Disadvantage of Nolvasan[®] is:

- Unknown efficacy against amphibian chytrid fungus. Experimental studies are needed.

Ethanol

Ethanol (70%) is useful for disinfection of equipment and instruments used in amphibian management. It is not recommended for enclosures or large equipment.

- Effective against both the amphibian chytrid fungus and ranaviruses (Langdon, 1989; Johnson et al., 2003). Experimental information for ranaviruses only examined a 2-hour contact time.

Ozone

Ozone is a strong oxidant with known antibacterial and antiviral properties. It has a track record of use for disinfection of wastewater in commercial aquaculture (Schuur, 2003; and OIE Manual of Diagnostic Tests for Aquatic Animals www.oie.int/eng/normes/fmanual/1.1.3_DISINFECTION.pdf) and will likely have application in facilities that must treat amphibian wastewater (see **Section 4.13**). It has not been specifically tested for activity against the amphibian chytrid fungus or ranaviruses.

Other Disinfectants

Other chemical disinfectants have been described for use against specific amphibian pathogens or are commonly used in commercial aquaculture or other animal husbandry settings.

- Povidone iodine: A commonly used topical antiseptic. Betadine[®] contains 10% povidone iodine, which is equivalent to 1% available iodine (Webb et al 2007). This product is potentially toxic to amphibians and is not recommended for widespread use in amphibian husbandry.
- Potassium permanganate, sodium chloride, and formaldehyde: these products are commonly used in aquaculture for their antimicrobial properties. Although they are readily available and inexpensive, they are potentially toxic and corrosive, and are not recommended for general use in amphibian management.
- Phenolic disinfectants: Retain more activity in environments contaminated with organic materials so may be useful for applications such as disinfectant foot baths. Can be very irritating to skin and have significant environmental concerns.

5.3 NON-CHEMICAL METHODS OF DISINFECTION

Heat

Application of heat is useful for disinfection of objects, organic substrate materials and water (both incoming water and wastewater) in amphibian facilities. Combinations of heat and pressure (e.g, autoclave or pressure cooker) are good for sterilization prior to use of materials in a facility or prior to disposal of contaminated solid waste.

The amphibian chytrid fungus is highly susceptible to modestly elevated temperatures. For example, exposure of pure cultures to a temperature of 32 °C (89.6 °F) resulted in death of the fungus by 96 hours of exposure time. Exposure to 47 °C (116 °F) resulted in death of the fungus within 30 minutes of exposure (Johnson et al., 2003). Elevation of environmental temperatures to these levels may be useful for elimination of this fungus from materials such as plants or other items that are hard to disinfect. Environmental temperature elevation has also been suggested as a treatment method

for amphibians that can tolerate higher environmental temperatures (Woodhams et al., 2003; Berger et al., 2004). A single study examined the effect of heat on cell cultures containing the EHNV *Ranavirus* of fish (Langdon, 1989). Exposure to 40 °C (104 °F) killed the virus at 24 hours. Exposure to 60 °C (140 °F) killed the virus within 15 minutes.

Dessication

The amphibian chytrid fungus is susceptible to complete drying (dessication). Death of the fungus occurred after 3 hours (Johnson et al., 2003). The fungus is known to remain viable in sterilized tap or deionized water for as long as 3–4 weeks and in sterilized lake water for up to 7 weeks (Johnson and Speare, 2003).

Brunner et al. (2007) found that dessication at 20 °C over 4 days inactivates the ATV *Ranavirus* in natural pond substrates, but that if the mud remained wet the virus remained active. Expanding this finding into the realm of captivity speaks to the importance of absolute dessication of the substrate, furniture, or enclosure—perhaps even for periods of a week or more.

Ultraviolet Light

Exposure to ultraviolet light has been used to disinfect water in aquatic systems and is known to have activity against bacteria and many viruses.

- Ultraviolet light at 1000mW/m² and wavelength of 254 nm was not effective against living cultures of the amphibian chytrid fungus in the laboratory (Johnson et al., 2003). The effect on free-swimming zoospores in water is unknown. UV light should not be relied on for disinfection of water against Bd zoospores until appropriate experiments have been performed.
- Ultraviolet light at 2.6 x 10⁴ uW.sec/cm² with a flow rate of 5000 L/h was effective against the Bohle iridovirus (*Ranavirus*) of frogs (Miocevic et al., 1993).

5.4 CLEANING AND DISINFECTION OF ANIMAL ENCLOSURES

General cleaning schedules for amphibian enclosures will vary depending on the species, the number of animals kept in the enclosure, the enclosure type, the types of substrates used, and the use of filtration systems.

- 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.
- Daily flushing of the substrate with water (where applicable) should be performed in all cages.
- 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.

- Organic substrates like sphagnum moss, peat, coconut husk, and soil-based substrates should be disposed of after use and not re-used in another enclosure.
- Frequency of water changes is dependent on the natural history of the particular amphibian species and the type of system used. Water testing for pH, ammonia, and nitrate are important parameters to monitor for many aquatic species, and can help to determine the need for partial or complete water changes (Browne et al., 2007; Pramuk and Gagliardo, 2008).
- Another factor for determining a cleaning schedule is whether or not a breeding program is initiated. Conditioning of some species may require aestivation. Some species are less apt to breed if they are disturbed. These and other species-specific requirements will be a consideration in the type and frequency of maintenance of an amphibian collection.

Disinfection of animal enclosures is performed:

- At the time of periodic substrate changes.
- As part of an approach to controlling an outbreak of an infectious disease in a captive amphibian population. For example, if there is an outbreak of chytridiomycosis it is important to eliminate the chytrid fungus from the enclosure as well as treating the sick animals.
- Before previously used enclosures are re-used with different animals.

Consult the table in Section 5.10 for details on the use of specific disinfectants including recommended concentrations and contact times.

Disinfectants are thoroughly rinsed from enclosure surfaces to avoid exposing amphibians to the disinfectant chemicals.

5.5 DISINFECTION OF EQUIPMENT AND TOOLS

Tools and equipment are cleaned and disinfected between use in different enclosures (e.g., a net used in Enclosure A is disinfected before use in Enclosure B). Consult the table in Section 5.10 for details on the use of specific disinfectants including recommended concentrations and contact times.

- Having multiple sets of equipment is helpful for efficacy in workflow (e.g., one set of equipment is disinfected while the other set is in use).
- For amphibian collections that have been established for long periods of time and that do not have ongoing infectious disease problems, it may be sufficient to simply thoroughly wash instruments or tools between different enclosures.

However, there is a risk for transmitting unrecognized pathogens between enclosures.

- For animals held at the ISOLATION level of biosecurity each long-term isolation room must have a dedicated set of equipment (e.g., nets, forceps, suction tubing, scrub brushes, sponges, etc.). This equipment does not leave the individual isolation room and is never used on a different group of animals. If enclosures or equipment from ISOLATION biosecurity rooms must be cleaned and disinfected at a location or sink that is used for the same purpose for animals in a different biosecurity level (e.g., BEST PRACTICES biosecurity), the location or area must first be thoroughly disinfected (see Chapter 4).

Thorough cleaning of equipment and tools to remove organic matter is essential prior to disinfection. Disinfectants are thoroughly rinsed from enclosure surfaces to avoid exposing amphibians to the disinfectant chemicals.

5.6 DISINFECTION AND STERILIZATION OF SUBSTRATES AND CAGE FURNITURE

There are a huge variety of substrates and cage furniture used in captive amphibian enclosures and each require different approaches to disinfection. As noted previously thorough cleaning and removal of organic material is necessary for effective disinfection.

Substrates and cage furniture made of plastic, glass or metal:

These materials are easy to clean and disinfect using chemical methods. Consult the table in Section 5.10 for details on the use of specific disinfectants including recommended concentrations and contact times.

Substrates made of natural or organic materials:

Natural or organic materials such as rocks, wood, or dead and dehydrated plant materials (e.g., sphagnum moss or coconut bark) as well as live plants are often essential components of amphibian enclosures, but are difficult to properly disinfect. Guidelines for disinfection of live plants are covered below in Section 5.7.

- 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 this web document: <http://www.colostate.edu/Dept/CoopExt/4dmg/Soil/sterile.htm>

From that same document, two effective methods of heat sterilization using microwave ovens are here reprinted:

1. Fill clean, quart-size plastic containers with very moist soil, perlite or cutting medium. Check the rims of the containers to make sure there is no aluminum of any kind because some yogurt containers come with a foil seal.

Use clean plastic yogurt containers with lids on for sterilizing soil. This is done using a temperature probe inside a carousel-type microwave oven, heating to 200°F and holding that temperature with the digital oven program for 20 minutes. Poke a hole through the plastic lids with a nail for steam ventilation. The temperature probe goes half way down into the soil through this hole in one of the containers. In a large microwave, up to 7 quart containers can be sterilized at a time, making this a very efficient way to heat sterilize soil. Allow to cool and tape over the hole in the lid to keep sterile until ready to use.

2. Place approximately 2 pounds of moist soil in a polypropylene bag. Leave the top open and place in the center of a microwave oven. Treat for 2½ minutes on full power of about 650 watts. After treatment close the top of the bag and allow the soil to cool before removing.

- Hardwood tree branches, wood and cork are porous materials that are difficult to properly disinfect. To minimize disease risk from these materials:
 - Consider the source before incorporating into amphibian enclosures. Avoid collecting materials from areas that are heavily exposed to native amphibians or areas known to be experiencing outbreaks of amphibian disease.
 - Application of modest heat is useful for eliminating the amphibian chytrid fungus (see Section 5.3). For smaller objects autoclaving or microwave heat sterilization as described above for substrates may be possible.
 - Wash in warm or hot water to remove as much dirt as possible. Avoid using soaps or detergents on porous materials.
 - Use of a dilute bleach solution (0.4% sodium hypochlorite) can be considered for disinfection after washing. Objects should be rinsed thoroughly after disinfection and allowed to dry for several days before use to ensure that amphibians are not exposed to bleach residues.
- In some cases, there are advantages to not sterilizing substrates. For example, leaf litter may be a source of microscopic prey essential to good success of captive management of a particular species. The disease risk must be considered on a case-by-case basis.

5.7 DISINFECTION OF LIVE PLANTS

Live plants can be a very important component of enclosures for captive amphibians. As described above for other substrates and cage furnishings, careful consideration of the origin of live plants can reduce disease risks. Selection of plants from a standpoint of amphibian facility biosecurity (especially amphibians kept in ISOLATION level of biosecurity) is discussed in Section 4.13. Strategies that can be used for plant disinfection and reduction of disease risk include:

- Plants should be removed from their pots. Rinse roots in clean freshwater to remove soil. Rinse plants overall to remove dirt or surface contaminants on stems and leaves. The plants may then be transferred to new, sterile soil and containers. Many plants may be rooted and grown hydroponically (in water), which is a preferable method where applicable.
- If the amphibian chytrid fungus is the primary concern, application of modest heat that is not lethal to many plants may be used for selective disinfection (see Section 5.3 above)
- Rinse plants in a dilute bleach solution ((0.4% sodium hypochlorite) dip for 10–15 minutes. Various plants will react differently, and some will not survive the treatment. For example, sturdy aroids like Pothos (*Epipremnum* spp.) and *Philodendron cordatum* will fare better than will begonias, ferns, and other plants with delicate stems or leaves.
- Whenever possible, obtain plants from local growers to minimize the risk of introducing non-native pathogens (e.g., a facility in Costa Rica should use Costa Rican plants, and not plants imported from the United States). A dedicated greenhouse, hydroponic, or aquatic plant growing facility that does not contain free-ranging amphibians is a good way to reduce the risks involved with using live plants.
- Where possible, especially for ISOLATION biosecurity situations, the use of plastic or silk plants is recommended. These are easily cleaned and disinfected with chemical or heat treatment.

5.8 DISINFECTION AND STERILIZATION OF WATER

Disinfection or sterilization of water may be necessary to ensure that water coming into a facility is free of amphibian pathogens or to ensure that wastewater exiting a facility does not contain pathogens that may pose a risk to native amphibians. These topics are discussed in Sections 4.14 and 4.16.

- If necessary, wastewater treatment from an amphibian facility can be accomplished by application of heat, ozone, or chlorine bleach (Schuur, 2003; Robertson et al., 2008; Krebs, 2008). Guidelines for aquaculture facilities are provided by the World Organization for Animal Health (OIE) (www.oie.int/eng/normes/fmanual/1.1.3_DISINFECTION.pdf).
- For disinfection of water by heat (either wastewater leaving the facility or incoming water) relatively low temperatures of 71 °C (160 °F) for 15–20 minutes are effective for the amphibian chytrid fungus and the EHNV *Ranavirus* in a laboratory setting. 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 (autoclave or pressure cooker) increases the effectiveness.
- Filtering water through one-micron (1µm) cartridge filters, available relatively inexpensively at hardware stores, is one method that may be successful at

removing zoospores 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 (see Section 5.3).

5.9 DISINFECTION AND BIOSECURITY IN THE FIELD

Concerns about the possibility of moving amphibian pathogens to new locations as the result of field research conducted on wild amphibians have led to a number of protocols for reduction of this risk:

- Amphibian Diseases Home Page:
www.jcu.edu.au/school/phtm/PHTM/frogs/field-hygiene.pdf
- Queensland Government Environmental Protection Agency:
www.epa.qld.gov.au/register/p02905aa.pdf
- California Center for Amphibian Disease Control:
www.ccadc.us/docs/DeconForProfessionals.pdf
- Association of Zoos and Aquariums Amphibian Husbandry Resource Guide (Chapter 2, by Kast and Hanna):
www.aza.org/uploadedFiles/Conservation/Commitments_and_Impacts/Amphibian_Conservation/Amphibian_Resources/AmphibianHusbandryResourceGuide.pdf

There are variations and sometimes contradictions between the different protocols, however, the basic principles of biosecurity for biologists working on wild amphibian populations are similar in all the protocols we have reviewed. Peer-reviewed publications including the addition of risk calculators to assist the biologist in making good biosecurity decisions have recently become available (St-Hilaire et al., 2009; Phillott et al., 2010). One of these risk calculators is available online: www.cefas.co.uk/4449.aspx

A summary of recommended field practices includes:

Definition of the field site:

The first precaution against the possible spread of disease among amphibian populations is careful definition of the field site or sites. Researchers should use natural and man-made boundaries to help define the sites. 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 (and transmission of disease) between sites.

On-site hygiene and biosecurity of equipment:

The use of disposable equipment discarded after use at a single site or on a single individual amphibian reduces the risk of spreading disease. All reusable

equipment, including footwear, should be disinfected between sites, or dedicated to a single site (e.g., a single pair of rubber boots is purchased for each field site and used ONLY at that site). Consult the table in Section 5.10 for details on the use of specific disinfectants including recommended concentrations and contact times.

- Footwear and other reusable equipment should be made of materials that are easy to clean and disinfect (e.g., rubber boots are better than leather hiking boots).
- Thorough cleaning of equipment is essential for removal of dirt and organic material prior to disinfection in the field. As noted in other sections, organic material inactivates many disinfectants. Scrub brushes and other implements to remove dirt should be part of the field equipment. If disinfectant solutions become contaminated with organic material or dirt they should be changed.
- The quaternary ammonium compounds (see Section 5.2) have been recommended for field situations because they are concentrated and easy to transport into field situations (Johnson et al., 2003; Webb et al., 2007).
- If disinfection is undertaken in the field, consideration should be given to the toxicity of chemicals to the environment. The quaternary ammonium compounds and Virkon® (see Section 5.2) are more environmentally friendly options compared to chlorine bleach (Johnson et al., 2003; Webb et al., 2007; Schmidt et al., 2009). If ranaviruses are a special concern Virkon® may have some advantages over the quaternary ammonium compounds (Bryan et al., 2009). Powdered bleach is another easily portable suggestion.
- Vehicles are 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 dirt and organic material 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.

Handling and collection of samples from amphibians:

When handling amphibians in the field, even within the same site, precautions should be taken to minimize the risk of transmitting pathogens between individual animals.

- Non-powdered disposable gloves are the best choice when handling amphibians. Powdered gloves should be rinsed free of powder. A new pair of gloves should be used for each animal. If gloves are unavailable, it is slightly preferable to use bare hands, and wash hands between handling different animals (Mendez et al., 2008).

- The greatest risk for spreading disease when handling amphibians occurs when animals are placed together in the same container or when containers are re-used without being disinfected. Do not re-use collecting bags and utilize a new one for each animal.
- Always handle animals as little as possible. Procedures that are quick, even if potentially painful, may cause less stress than longer procedures.
- Animals should only be released at the site of capture and any sick or dead amphibians found should be preserved in 10% buffered formalin solution and submitted for disease diagnosis (see Chapter 9).
- Instruments used for sample collection should be disinfected between use on different animals. For surgical instruments (e.g., scissors) and weighing equipment 70% ethanol is rapidly acting against the amphibian chytrid fungus (Johnson et al., 2003).
- Although mentioned in some amphibian handling protocols the use of iodine-based compounds for sanitizing the skin prior to procedures such as toe-clipping or microchip implantation is not recommended because of toxicity concerns. Potential substitutes include 0.75% chlorhexidine or 2mg/L benzalkonium chloride (Wright, 2001).

5.10 TABLE OF DISINFECTANTS

It is important to remember that disinfectant concentrations and contact times established in trials conducted *in vitro* may not be effective when directly transferred to practical situations, since organic matter is detrimental to the efficacy of a range of disinfectants (Johnson, 2003; Webb et al., 2007). Recommendations for field disinfection are generally adapted from *in vitro* studies by arbitrarily increasing the disinfection concentration or contact time beyond the *in vitro* levels. For example, Webb et al. (2007) recommend using twice the minimum *in vitro* concentrations for field disinfection.

Disinfection strategies suitable for killing the amphibian chytrid fungus and ranaviruses: All concentrations and contact time are *in-vitro*. Information derived from (Johnson et al. (2003), Webb et al. (2007), Langdon (1989), Miocevic et al. (1993), Bryan et al., 2009) and Phillott et al. (2010). Table is adapted from Kast and Hanna (2008).

Application	Disinfectant	Concentration	Contact Time	Target Pathogen
Disinfecting surgical equipment and other instruments (e.g., scales, calipers)	Benzalkonium chloride	1 mg/mL	1 min	Chytrid Fungus
	Ethanol	70%	1 min	Chytrid Fungus
	Ethanol	70%	2 hr	Ranaviruses
	Virkon [®]	1% (10mg/ml)	1 min	Chytrid Fungus Ranaviruses
	Chlorhexidine	0.75–2%	1 min	Ranaviruses
Disinfecting equipment, enclosures, footwear and vehicles	Sodium hypochlorite (bleach)	1% sodium hypochlorite	1 min	Chytrid Fungus
	Sodium hypochlorite (bleach)	0.4% sodium hypochlorite	10 min (minimum)	Chytrid Fungus
	Sodium hypochlorite (bleach)	3% sodium hypochlorite	1 min (wet surfaces)	Ranaviruses
	Didecyl dimethyl ammonium chloride (DDAC) 8–12% concentrated solution	1 in 1000 dilution	0.5 min	Chytrid Fungus
	Trigene [®]	1 in 5000 dilution	1 min	Chytrid Fungus
	F10 [®]	1 in 1500 dilution	1 min	Chytrid Fungus
	Virkon [®]	1% (10 mg/mL)	1 min	Chytrid Fungus Ranaviruses
	Chlorhexidine	0.75–2%	1 min	Ranaviruses
	Complete drying (dessication)		>3 hr	Chytrid Fungus
	Heat	60 °C	15 min	Chytrid Fungus Ranaviruses
	Heat	37 °C (Section 5.3)	4 hr	Chytrid Fungus
	Sterilizing UV light	(See Section 5.3)		
	Disinfecting cloth (e.g., bags, clothes)	Hot wash	60 °C or greater	30 min

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CHAPTER 6

QUARANTINE

6.0 INTRODUCTION

Quarantine of new (incoming) animals before introduction into an established amphibian collection (e.g., animals in zoos or established amphibian survival assurance colonies) is a standard practice in well-managed animal facilities. The most basic purpose of a quarantine program is to prevent transmission of significant pathogens present in the incoming animals to established collection animals that are presumed to be free of these pathogens. The process of quarantine involves:

- Holding new animals in isolation from the established animal collection.
- Adapting new animals to a new situation and husbandry practices.
- Assessing health threats present in both the new animals as well as animals in the established collection.
- Mitigating or resolving health threats identified in the new animals.

This chapter focuses on quarantine for new animals entering established amphibian collections. There is considerable overlap in procedures and philosophy with the process of long-term isolation used for captive animals or their progeny that are destined to be released to the wild (see Chapter 4). General references for the development of amphibian quarantine programs include: Wright and Whitaker, 2001; Lynch, 2001; Ferrell, 2008).

6.1 GOALS AND DEVELOPMENT OF QUARANTINE PROGRAMS

Each institution or facility that manages amphibians in captivity should create and follow an amphibian quarantine protocol. Quarantine protocols are created and customized for each institution based on the types of species housed in a facility; the resources available to the institution; the unique needs and requirements of recovery programs for individual species or species assemblages; or in response to changes in disease risk assessments.

When drafting a quarantine protocol, the most important goals are to:

- Protect the health of the established collection animals by reducing the risk that amphibians new to the animal collection will introduce an infectious disease.
- Follow guidelines for long-term isolation of animals that will be reintroduced to the wild (see Chapter 4).

- Properly acclimate wild-caught animals in quarantine to captive husbandry practices (e.g., diet, local water qualities, social environment, enclosure design, behavioral husbandry routines, and provision of enrichment).

Additional goals that can be accomplished by a well-designed quarantine program are:

- Documentation of the general health, infectious disease and parasite (including commensal organisms) status of new animals as they enter quarantine. This provides important information for informed management of captive or wild amphibian populations.
- Perform a disease risk assessment to determine if new animals are suitable for their ultimate role (e.g., for exhibit, research, breeding in a survival assurance colony or reintroduction to the wild).
- Provide a means for unique identification of individual animals. If identification of individuals is impractical, develop plans to manage a colony in a manner that preserves the goals of the program (e.g., a survival assurance colony needs to be managed to conserve and maintain genetic diversity). Methods for individually marking animals are reviewed in Appendix 1.
- Collect normal reference values for clinical pathology (e.g., hematology and clinical biochemistry) that may be helpful for medical management. This information does not exist for the vast majority of amphibian species.

Important information and resources that may be required to develop a quarantine protocol include:

- Definition of the role of the new amphibians entering the collection (e.g., exhibition, research, or survival assurance colony).
- Knowledge of institutional practices that are in place to detect and control infectious diseases in the established amphibian collection. For multi-institutional amphibian recovery projects this information should be available from all participating institutions.
- Is a consulting or staff veterinarian available to guide medical assessment of both incoming animals and established collection animals?
- Is diagnostic laboratory support available for health screening? Because necropsy and histopathology of animals that die can be an important part of disease risk assessment, the availability of a consulting veterinary pathologist should be considered.
- Knowledge of national and international laws and policies that pertain to the species housed in the facility (e.g., CITES) or that regulate infectious diseases in the context of import, export and other movements of captive amphibians (e.g., OIE). All necessary permits and reports should be reviewed to ensure that all are in order. The World Association for Animal Health (OIE) guidelines for mitigating the risk of exporting and importing the amphibian chytrid fungus and ranaviruses

should be reviewed:

http://www.oie.int/eng/normes/fcode/fcode2008/en_chapitre_2.4.1.htm

- What financial resources are available to fund quarantine activities? Quarantine costs increase with the level of disease risk. Costs of quarantine may include, but are not limited to: staff training, labor, laboratory testing (including necropsy and histopathology), shipping of animals and of samples for laboratory tests, feeding of animals, medications and medical supplies, utilities, materials for disinfection and costs of maintaining long-term isolation.

6.2 RISK ASSESSMENT IN AMPHIBIAN QUARANTINE

Determination of essential elements of the quarantine process such as the length of the quarantine period; the types and extent of laboratory testing needed for health assessment; and the extent of veterinary treatment needed for new animals is dependant on a qualitative risk assessment. This assessment is performed for each group of new animals that enters quarantine. In general, new animals that have an overall LOW risk require shorter quarantine periods and less diagnostic testing, whereas, animals with a HIGH risk need a longer quarantine period and more extensive or repeated diagnostic testing.

Components of the quarantine risk assessment are:

- **The source of the incoming animals.** New animals vary significantly in their potential exposure to important amphibian pathogens. For example, animals that have been maintained at a facility that mixes amphibians of different species, different geographic locations and different sources in a single room or water system (also known as a “mixed” or “cosmopolitan” collection) can be at a higher risk of carrying infectious agents of concern. In contrast, animals that come from a facility that is dedicated to a single species or animals housed in long-term isolation within a cosmopolitan facility (see Chapter 4) may be at a lower risk of carrying infectious agents of concern. For both situations, the level of risk is lower if new animals come from a source that is trusted; is known to have good facility hygiene and biosecurity practices; and can provide health history information (see below).
- **The health history of incoming animals.** New animals that come from well-managed captive populations in zoos or aquariums (and some wild amphibian populations) often have a health history developed by long-term veterinary care and disease surveillance. This surveillance should include routine necropsy and histopathology of animals that die (see Chapter 9). If available, the health history can be reviewed for the presence or absence of specific health problems in the source population and aids significantly in developing quarantine plans. Animals that lack a health history (e.g., some animal dealerships or wild amphibian populations) are considered to be a higher disease risk.

As disease issues are identified during review of the health history, a level of risk (HIGH, MODERATE, or LOW) is determined. Those disease issues with HIGH or MODERATE risk require the most extensive laboratory testing or treatment during quarantine to reduce disease risk to established collection animals. Specific issues identified during a health history that indicate a HIGH disease risk include:

- Significant unexplained mortality or illness in the source population (e.g., more than 10% of the adult population dies or shows evidence of disease within 30 days). The concern in this situation is introduction of new undescribed or difficult to diagnose infectious disease agents to an established amphibian collection.
- Recent identification (within 6 months to 1 year) in the source population of pathogens known to be population limiting to wild or captive amphibian populations (e.g., amphibian chytrid fungus or *Ranavirus* infection).

Specific issues identified during a health history that indicate a MODERATE disease risk include recent identification (within 6 months to 1 year) in the source population of infectious diseases that are known to cause significant clinical disease, but are not necessarily known to be population limiting (e.g., mycobacteriosis or rhabditiform nematode infection).

Specific issues identified during a health history that have a LOW risk include those infectious agents that are usually considered to be opportunistic infections (e.g., *Aeromonas hydrophila* or *Saprolegnia*).

- **Role of the New Animals.** The ultimate role of the new animals entering a collection as well as the role of established collection animals that will be exposed to the new animals has an impact on disease risk assessment. For very valuable amphibian survival assurance populations (e.g., a critically endangered species or a species for which there are few survival assurance populations), it may be prudent to treat all new animals as a MODERATE to HIGH risk because of the greater consequences of inadvertently introducing an important infectious disease. This level of caution may not be necessary if the source population of animals has an extensive health history that is considered to be LOW risk (see above).

6.3 QUARANTINE FACILITY AND QUARANTINE STAFF CONSIDERATIONS

Location and Access to Quarantine Room or Facility

Animals in quarantine should be kept in a building or room that is separate from the established amphibian collection or survival assurance colony. Quarantine rooms or facilities should be designed using guidelines for Long-Term Isolation facilities described in Chapter 4).

The designated quarantine space must be secured to prevent unauthorized access, be it accidental or purposeful, which could inadvertently result in the movement of materials contaminated with infectious agents from the quarantine animals to the established collection animals.

Order of the Quarantine Husbandry Routine

Under ideal conditions, new animals in quarantine are cared for by staff members dedicated only to quarantine animals that do not also care for amphibians in the established collection. However, in most institutions, it is not possible to have staff members that only care for animals in quarantine. The traditional recommendation in this situation is to have regular staff members care for the new animals in quarantine last on each work day and only after caring first for animals in the established collection.

- Staff members should not move back-and-forth during the day between areas containing quarantine animals and established collection animals. This is the preferred approach that minimizes the chance of accidentally moving amphibian pathogens from quarantine animals to established zoo collection or survival assurance population animals.
- If during the work day, staff members must move between caring for new quarantine animals to established collection animals it is very important that guidelines for quarantine staff wardrobe and footwear be followed (see below).
- An exception to the guideline of caring for new animals in quarantine last in the day occurs if the new animals must be maintained in Permanent Isolation (Chapter 4, Biosecurity and Permanent Isolation). Animals that are kept in Permanent Isolation will ultimately produce animals for reintroduction into the wild. For these animals, there is an added concern about the potential for introducing novel infectious agents from a general mixed zoo amphibian collection to vulnerable wild amphibian populations as the result of the reintroduction program. Therefore, new animals in quarantine that require Permanent Isolation are cared for BEFORE caring for animals in the general amphibian collection.

Quarantine Staff Wardrobe

To reduce the risk of spreading pathogens from new animals in quarantine to animals outside of quarantine (this includes established collection animals as well as native amphibians) and to reduce the risk of introducing pathogens from outside to new animals in quarantine, there are specific recommendations for quarantine staff wardrobe including:

- **Footwear dedicated to each quarantine room.** Shoes or boots worn in the quarantine area should not be used in areas that house established collection animals. Likewise, shoes worn in areas with established collection animals should not enter the quarantine room. The use of disposable surgical foot

coverings or plastic foot coverings (e.g., ShuBee brand) for each room is a less desirable alternative to dedicated footwear. Disinfectant footbaths are not a good replacement for dedicated footwear (see Section 4.10).

- **A uniform or protective clothing should be dedicated to each quarantine room.** A uniform worn in the quarantine area should not also be worn in areas that house established collection animals without first being laundered. Similarly, a uniform worn to care for established collection animals should not be used to care for animals in quarantine. Alternatives to a uniform include a full-length laboratory coat, surgical scrubs, coveralls or disposable clothing (e.g., Tyvek brand).
- **Dedicated disposable gloves must be worn while working directly with amphibians or amphibian enclosures.** Use of dedicated gloves may be needed per individual animal enclosure, per species or species assemblage depending on disease risk. This is especially true if animals from different original sources are housed in the same quarantine room. There have been toxicity concerns for tadpoles with commonly used latex, vinyl and nitrile gloves (Greer et al., 2009). Staff members should wash their hands and arms frequently and especially when moving between different rooms.

6.4 ANIMAL HUSBANDRY CONSIDERATIONS IN QUARANTINE

All in/All out

All animals in a quarantine room should enter a quarantine period at the same time and leave quarantine at the same time (“All in/All out”). Allowing different animals to enter or leave the same quarantine room at different times (“staggering” of quarantine) increases the risk that an important pathogen will be introduced to all of the animals in the quarantine room and go undetected prior to release of the animals that first entered quarantine. This, in turn, increases the risk of introducing an important infectious disease agent to the established amphibian collection.

- If new animals must enter a quarantine room prior to completion of an already ongoing quarantine period, the length of the quarantine period should be extended for the animals that entered the quarantine room first.
- No animals should be released from the quarantine until the animals that entered quarantine last have finished their quarantine period.
- Releasing all animals from the quarantine room at the same time has the important advantage of allowing the quarantine space and enclosures to be completely cleaned and disinfected between incoming quarantine groups.

Biosecurity in Quarantine

All animals in a single quarantine room should have the same ultimate role (Ark, Rescue or Supplementation; Conservation Research or Education) and should have a history of being maintained at a level of biosecurity (ISOLATION or BEST PRACTICES) appropriate for that role (see Section 4.4 for an explanation of biosecurity levels).

- Animals that are destined to enter a survival assurance colony with the purpose of returning animals or progeny to the wild (e.g., animals that have an Ark, Rescue or Supplementation role and are kept in Isolation) should never be housed in the same quarantine room with animals that have not been previously housed in Long-Term Isolation. These animals should also be maintained as a single species or species assemblage (an amphibian faunal group that is from the same source population) per quarantine room.
- As an example, it would not be desirable to house a Wyoming toad (from the USA) that is part of a survival assurance population that produces animals for reintroduction to the wild in the same quarantine room as a White's tree frog (from Australia) that is destined for use as an education or display animal.

During quarantine, directional routines of husbandry practices that minimize cross contamination between different animal enclosures should be followed. These are described in detail in Section 4.11.

Enclosures and Cleaning

Enclosures that are easy to clean and disinfect are recommended for quarantine facilities. Enclosures should be as simple as the husbandry requirements and natural history of the species will allow, while still encouraging natural behavior as the species adapts to captivity (e.g., incorporate refugia, substrates or other furnishings that stimulate natural behavior). Adaptability and appreciation for the natural history of the species are keys to a successful husbandry protocol in quarantine. For instance, not all species will tolerate a stark enclosure with minimal furnishing (ideal for cleaning and disinfection) and these very sensitive animals may require complex naturalistic enclosures during the quarantine period. Some territorial species (e.g., dendrobatid poison frogs) may need to be maintained with visual barriers between enclosures.

- Enclosures should be easily accessed and made of non-porous glass, fiberglass, or plastic materials that are easily cleaned and disinfected.
- After a group of animals has completed quarantine enclosures are completely disassembled and disinfected prior to re-use. This includes filtration systems used for totally aquatic amphibians. Guidelines for disinfection of enclosures are provided in Section 5.4.

Substrates and cage furniture or decorations for quarantine enclosures should be disposable or should be made of materials that can be completely sterilized after use. Suggestions for substrates include:

- **Sphagnum moss.** This material is relatively inexpensive, has high water retention, and has antibacterial properties.

- **Pea gravel.** A thin layer of pea gravel at the bottom of the enclosure to aid with drainage, or a false bottom, with a layer of sphagnum to allow the enclosure to be flushed thoroughly and frequently.
- **Plastic mats.** Various sorts of plastic mats (e.g., non-slip liners for toolboxes) or sheets of perforated PVC can be used as a substrate. However, they must be thoroughly scrubbed and disinfected between usage because biofilms may easily accumulate among the textured surfaces.
- **Paper towels.** These are convenient and hygienic, but do dry out (desiccate) rapidly and may require increased diligence on the part of staff members. Paper towels should be non-bleached and non-imprinted, to avoid exposure of amphibians to toxic chlorine or dyes.
- **Plants.** Can be very important for some amphibians as a refuge or as a resting or sleeping substrate. Plastic or artificial plants are useful because they are inexpensive and easily disinfected or discarded after a group of animals is released from quarantine. If live plants are used in quarantine, plants that do require substrate (e.g., Pothos, epiphytes), or that can have substrate maintained away from the animals, are preferred. Plants should not be re-used after the quarantine period is completed.

Because of concerns about introduction of infectious disease agents, it is recommended that substrates and furniture are never collected from outside, especially from areas where they have been in contact with other amphibians. Guidelines for biosecurity practices that reduce the possibility of introducing important amphibian infectious diseases from enclosure substrates and plants are provided in Section 4.13.

General cleaning (removing feces and unconsumed food items along with flushing substrate with water as possible), of all cages should be performed daily. Complete replacement of substrates should be performed weekly if possible. These guidelines are important because some parasites (e.g., rhabditiform nematodes) can complete a full life cycle in as little as 48 hours. For species that are easily stressed in a captive environment, frequent cleaning or replacement of substrates may be too disruptive. In these cases consideration may be given to less frequent cleaning depending on the types of parasites present and estimated parasite burden of the species.

Aquatic Enclosures

Totally aquatic amphibians in quarantine will require special attention to water quality. This requires either a water filtration system or frequent water changes (e.g., “dump and fill”). It is helpful to be able to monitor water quality parameters such as ammonia, nitrate, nitrite and pH.

- If a filtration system is used it is necessary to have an established “biological filter” of specialized bacteria to breakdown ammonia by-products of nitrogenous wastes created by animal excretions, uneaten food items, or other organic

material. This usually requires a system to be running for 2–3 weeks prior to new animals entering quarantine (advanced planning is required).

- For biosecurity reasons, if bacterial colonies from existing tanks in the facility are used to start a biological filter, these colonies should not be selected from water systems or tanks that have previously housed amphibians.
- Systems with newly established biological filters may still require frequent water changes to maintain water quality as bacterial colonies can be inefficient. This is especially true if antibiotics are used in the water during the quarantine process.
- When quarantine is completed, any filtration system should be disinfected along with the enclosure, to prevent spread of organisms that may be harboring within the filter matrix. In other words, the same filtration system should not be used for more than one group of animals in quarantine.
- An alternative approach is to avoid filtration systems altogether, and simply adopt a regular “dump and fill” approach for all aquatic enclosures during the course of the quarantine period.

Handling of Waste and Wastewater

There are concerns that waste materials (e.g., soiled substrates) or wastewater from captive amphibian facilities can serve as a source for introduction of important infectious disease agents to wild amphibian populations. Similarly, wastes from quarantine animals might serve as a source of infectious agents for established collection animals. Therefore, protocols should be established that ensure that waste materials do not come into contact with local ecosystems or with other animals that are outside of the quarantine facility. Waste and wastewater treatment may be required under World Organization for Animal Health (OIE) guidelines for importation of amphibians from countries not declared to be free of the amphibian chytrid fungus or ranaviruses: www.oie.int/eng/normes/fcode/fcode2008/en_chapitre_2.4.1.htm General guidelines for wastewater and solid waste disposal are available in Sections 4.13 and 4.14.

Diet and Feeding of Animals in Quarantine

Wild caught insects can be an excellent source of nutrition, especially for animals that are not yet adapted to captive diets, but do carry a risk for introducing an infectious disease agent or chemical contaminant. There can also be a slight risk for disease introduction with the use of prey items from commercial suppliers. General recommendations for biosecurity practices that reduce the possibility of introducing important amphibian infectious disease agents from food sources are provided in Section 4.17.

Reducing Stress

Quarantine programs must sometimes consider the balance between goals of the quarantine and the needs of the animal (e.g., need to avoid stress in sensitive species). Very sensitive species may not survive the quarantine process unless attempts

are made to accommodate for their special needs. Flexibility and individual disease risk assessment for each group of animals that enters quarantine can be helpful in this regard. Animal stress can be reduced by:

- Minimizing the frequency that animals are handled or disturbed. As much as possible physical examinations, collection of samples for disease testing and other activities should be combined into a single activity. Implementation of automation in husbandry routines (e.g., pre-plumbed misters on a timer to maintain humidity in the enclosure) can also be helpful.
- Offer appropriate refugia for hiding and encouraging natural behaviors of the animals. Use of easily cleaned or disposable materials (e.g., plastic plants and cut pieces of PVC pipe) allows for reduced animal stress while allowing for easy disinfection.
- Providing diets, lighting, water composition, temperatures, space requirements (horizontal vs. vertical space), substrates and animal density in a manner consistent with the natural history of the species can significantly reduce low grade chronic stress. This is especially true for sensitive species or for species for which there is limited captive husbandry experience. The latter is an increasingly common scenario as more amphibian survival assurance populations are established. In many cases it may be desirable to establish husbandry protocols on a closely related “surrogate” species before acquiring an especially sensitive or valuable species.

6.5 LENGTH OF THE QUARANTINE PERIOD

The length of time that new animals spend in a quarantine period is determined by several factors. These factors include:

- **The results of a quarantine risk assessment** (see Section 6.2). Animals that are determined to be of LOW disease risk require less diagnostic testing and veterinary observation and therefore will have shorter quarantine period.
- **The overall health of the animals in quarantine.** This determined by veterinary evaluation, diagnostic laboratory testing, and by the occurrence of significant illness or deaths in the animal group. If significant disease problems are identified or if other criteria for release of animals from quarantine (see below) are not met, the quarantine period will be longer.

The minimum suggested length for an amphibian quarantine period is 30 days. This short time period is most appropriate for LOW to MODERATE risk quarantine situations that do not require extensive diagnostic laboratory testing and when animals meet all other criteria for release from quarantine. For most MODERATE to HIGH risk quarantine situations, a minimum quarantine period of 60 to 90 days is suggested. This longer quarantine period allows for careful observation of the quarantine animals for

evidence of disease and allows time to receive the results of necessary laboratory testing.

Criteria for release of animals from quarantine after the minimum quarantine period are:

- A successful physical examination by a veterinarian or other trained personnel. If practical, detailed physical examination should be made of each individual animal in the group. See Section 6.6 for details on performing a physical examination. If a large group of animals is in quarantine and physical examination of individual animals is impractical, visual examination of as many animals as possible is acceptable. Attempts should be made to perform more detailed physical examination and laboratory screening on at least 10–30% of the individuals.
- Individual animals have maintained a minimum body condition score (BCS) of “3” for at least 14 sequential days prior to the planned date of release from quarantine. See Section 6.6 for details on body condition scoring.
- The quarantine group of animals has had no unexplained mortalities for at least 14 days in a row prior to the planned date of release from quarantine.
 - If pathology services are available, final histopathology results should be reviewed from necropsies of animals that died during the quarantine process or that were culled (euthanized) for disease surveillance purposes during quarantine. If an infectious disease process has been identified, the quarantine period is extended until the issue has been resolved.
- Final results of any laboratory testing (e.g., PCR for amphibian chytrid fungus) are negative for important amphibian pathogens. In some cases, results of tests may not be available in a timely manner (e.g., if PCR samples must be exported to another country for analyses); in such cases, careful consideration may be given to alternatives such as prophylactic treatments (see Section 8.1). If an infectious disease process has been identified, the quarantine period should be extended until the issue has been resolved.
- Treatments for parasitic infections often influence the length of a quarantine period. If a determination is made to treat detected parasites (see Section 8.4), parasite treatment must be completed and appropriate post-treatment parasite screening must be performed and target parasite reductions must be attained prior to quarantine release. This process may prolong quarantine by several weeks or more and more than one round of treatments may be required.
- If sick animals have been identified in the quarantine group, all health issues should have been resolved (either by death and necropsy or return to an apparently healthy state) for at least 14 days before the planned date of release from quarantine. This number is somewhat arbitrary and may be adjusted according to the judgment of the consulting veterinarian or other trained personnel.

- Attempts should be made to determine if sick animals are afflicted with an infectious process or if illness is due to maladaptation to quarantine or captivity. If infectious disease can be successfully excluded, consideration should be given to releasing the animal from quarantine to a permanent enclosure that may be better suited to the animal's needs.

6.6 MEDICAL CONSIDERATIONS IN QUARANTINE

Careful observation and recording of individual health of animals in quarantine is the conceptual core of the entire program. Observations of individuals may detect disease problems before animals are allowed to leave the quarantine facility and reduces the likelihood that new infectious diseases will be introduced to the established collection.

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 ISIS (International Species Inventory System) 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 into quarantine, source/identification of sire and dam, individual institution accession number, and any other identifiers such as studbook numbers, microchips and bands. Group animals are given a group accession number with the aforementioned information provided, as is practical. Quarantine notes should contain information about means to individually identify animal (if applicable) especially if it depends on physical markings, or weights (see section below on body weights for individuals and groups).
- **Quarantine notes.** All animals, whether as individuals or groups, should have some daily notation in the quarantine portion of the record made as to presence or absence of at least the following factors: feces, urine, food intake, normal behavior and attitude. There is little difference between keeping medical record formats between groups or individuals as long as specific population changes are well documented (i.e., three out of five animals appear dehydrated or are lethargic versus simply saying “some animals appear sick”).
- **Clinical notes by veterinary staff.** This section includes notes and observations on clinical diagnoses, anesthesia and medication prescriptions.
- **Laboratory results.** Laboratory results from parasitology, serology/molecular diagnostics, microbiology, and necropsy/histopathology.

Physical Examination

Under ideal conditions individual animals should receive a physical examination at least three times during quarantine. Examinations are performed by a veterinarian or other personnel that have received training in health evaluation.

- Examinations take place at the times of entry into quarantine, midpoint in the quarantine period, and before exit from quarantine. Handling time during examinations should be limited to avoid stress in sensitive species, especially during entry to quarantine. Everything required for examination or identification should be organized prior to examination, so no time is wasted while the animal is in hand.
- Examinations can vary in their duration and extent. In large groups of animals, a subset of the population (10–30%) is examined to assess the health of the population.
- Special attention is given to body and skin condition, coelomic palpation, and an oral examination. Transillumination (use of a bright focused light to highlight internal coelomic structures) can be useful for visualization of coelomic structures, especially investigating coelomic diseases such as detect coelomic diseases such as ascites, neoplasia, granulomatous processes, or masses.
- All physical or behavioral abnormalities are documented in the medical record. Common external abnormalities that should be noted include missing limbs/digits, ulcers, or abnormal skin color/texture.
- Animals are assessed for body weight and body condition score (see below)
- Features useful for individual identification of animals should be noted (e.g., unique coloration or skin patterns). Digital photographs can be obtained of individuals for later reference in case of illness or for use in comparing to an ideal body condition or morphology for the species.

Animal Identification

Permanent identification of individual animals is a critical tool for captive animal management especially as it pertains to maintaining genetic diversity in reproductive efforts. In quarantine situations individual animal identification is helpful for:

- Monitoring of body weights and body condition scores
- Epidemiologic investigations by identifying disease carriers and sites of exposure to pathogens or other harmful agents.
- Assessment of the effectiveness of manipulations in captive husbandry parameters such as diet, caging, and environmental parameters. These factors are more easily analyzed with unique identification of animals.

The negative aspects of individual identification are the costs of identification equipment and placement, secondary trauma/infection from the marking mechanism,

and the potential loss and need for replacement of the ‘permanent’ identification marker. See Appendix I for details on animal identification methods.

Body Weights and Body Condition Score

As noted in earlier sections individual animal body weights and body condition scores are important components of health evaluation in quarantine. Notes should be kept as to the general appearance of the body condition during quarantine with special attention given to body condition scores on entry into quarantine and before exit from quarantine.

The 5 Point Scale BCS System

Score	Description
1	Emaciated—Lumbar vertebrae, pelvic bones and all body prominences evident from a distance. No discernible body fat. Obvious absence of muscle mass.
2	Thin—Tops of lumbar vertebrae visible. Pelvic bones less prominent. Obvious waist and coelomic tuck.
3	Moderate—No prominent pelvic bones. Coelom tucked up when viewed from side.
4	Stout—General fleshy appearance. Noticeable fat deposits over lumbar spine, limbs, and tail base. Coelomic tuck may be absent
5	Obese—Large fat deposits over chest, spine, and tail base. Coelomic tuck absent. Fat deposits on neck and limbs. Coelom distended

Body weights for individuals should be taken when animals enter quarantine and then on weekly basis thereafter.

- A precision scale (0.01 g for small animals) should be used for weighing animals. The same scale should be used for each time an animal is weighed to reduce confounding influence of variation between scales.
- Weights for individual animals can fluctuate. Staff unaccustomed to working with very small animals should be advised that a natural act such as defecation or urination can produce a seemingly large and rapid loss in weight; amphibians frequently urinate when handled. As a guideline, "significant weight loss" of an amphibian may be on the scale of >10–15% of previous body weight/week, and should be considered as a possible indicator of abnormal health. Changes in body weight should be evaluated against visual assessments of body condition score.
- Healthy animals should usually maintain body weight or possibly gain weight during quarantine. Maladaptation to captivity or to quarantine conditions is a consideration for weight loss.

- For large groups of animals, examination of the group's median weight and the maximum and minimum values can be used to estimate a trend for overall weight loss or gain within the group.
- A final exiting weight should be taken before exit from quarantine. For large groups of animals, weights can be taken on all animals weekly to biweekly with the values recorded under a group identification number. Examination of the group's median weight and the maximum and minimum values can be used to roughly estimate a trend for overall weight loss or gain within a group. The same concerns exist for overall weight loss within a group at quarantine exit as exist for release of an individual animal with weight loss.

6.7 LABORATORY TESTING AND DISEASE TREATMENT IN QUARANTINE

Laboratory diagnostic testing and prophylactic treatment are useful and sometimes essential for the medical evaluation and risk assessment of animals in quarantine. Specifically, targeted diagnostic testing or treatment can:

- Detect and reduce the impact of pathogens that are known to cause significant mortality in captive populations before quarantined animals are introduced to the general collection or to a survival assurance colony.
- Assist in the development of captive populations that are free of specific pathogens (e.g., amphibian chytrid fungus). Such specific pathogen-free populations are necessary for programs that intend to reintroduce animals to the wild, or intend to distribute amphibians to other zoological institutions.
- Identify unsuspected disease problems in source populations.

It is difficult to create a single disease testing or treatment protocol for use in all amphibian quarantine situations. The decision to use different types of diagnostic testing is situational and depends on factors such as:

- **Quarantine Disease Risk Assessment.** See Section 6.2 for a detailed description of the quarantine risk assessment.
- **Availability of laboratory or veterinary support.** Not all facilities that keep captive amphibians have access to laboratories and veterinarians that can perform diagnostic tests and interpret laboratory results. In these situations there is greater reliance on prophylactic treatment for disease agents of concern (e.g., amphibian chytrid fungus or rhabditiform nematode parasites); use of extended quarantine periods to observe animals for signs of illness; and reliance on physical examination to assess animal health.

Common laboratory diagnostic or treatment methods that might be used in quarantine situations include:

- Necropsy (postmortem examination).

- Laboratory examination of feces and treatment for internal parasites.
- Testing and/or prophylactic treatment for the amphibian chytrid fungus.
- Testing for ranaviruses.
- Obtaining samples for clinical pathology (hematology and blood chemistry).

These methods are discussed in more detail in the sections below.

6.8 NECROPSY

The necropsy (postmortem examination) is a powerful tool for disease screening of animals in quarantine and ideally should be performed on all animals that die. Amphibian necropsy procedures are described in detail in Chapter 9.

- Necropsy with histopathology is a test that is not specific for any one type of disease and therefore has the potential to detect a wide range of infectious diseases (e.g., chytridiomycosis or *Ranavirus* infection); clinically important parasitic infections (e.g., heavy loads of rhabditiform nematodes); and nutritional problems (e.g., metabolic bone disease), among many other disease conditions that can be encountered in captive amphibians.
- Necropsy and histopathology is the diagnostic method that most new or unexpected disease problems—for which there is no specific diagnostic test available or validated for amphibians—will be detected.
- Frequently, results from a necropsy are used to quickly identify the major health problems in a quarantined group of animals and help to focus laboratory diagnostic and treatment efforts during quarantine.
- Because many disease conditions are undetectable to the naked eye or look similar to other conditions (e.g., a frog with red skin could have a bacterial infection, a fungal infection or a viral infection), histopathology by a veterinary pathologist is frequently necessary.

Culling of animals (by euthanasia) for diagnostic sampling and histopathology may be used as part of a disease surveillance program in quarantine. Animals selected for culling should be humanely euthanized and details for this procedure are provided in Section 8.6. Situations where culling is especially useful include:

- Investigation of a disease outbreak or unknown mass mortality in quarantine.
- Culling can also be considered if there is a large group of animals in quarantine and there is very little information known about the important disease problems in the species or if the animals have been obtained from a source that is considered to be of HIGH risk for infectious disease introduction. Also, for programs bringing wild amphibians into survival assurance colonies, disease surveillance programs that include culling and necropsy, either of the target species or of surrogate sympatric species (or both), are useful for determining important disease risks and problems prior to establishing the captive program.

In these situations, culling offers the best chance of obtaining high quality diagnostic specimens that have minimal artifacts from postmortem decomposition (autolysis).

The decision to use culling of animals for disease surveillance can be controversial. Therefore it is important to have agreement from all members of an amphibian conservation program about the need for culling and identification of those animals that will be removed for this purpose.

6.9 TESTING AND TREATMENT FOR INTERNAL PARASITES

Screening for internal (usually intestinal) parasites is a standard veterinary practice for quarantine. Screening is usually performed by laboratory examination of the feces for characteristic parasite eggs or other life-stages. Some degree of internal parasitism is expected for wild amphibians and the host usually tolerates endemic parasites very well.

- Under captive husbandry conditions infections with some types of parasites, especially the rhabditiform nematodes such as *Rhabdias* spp. (the amphibian lungworm) or *Strongyloides* spp. (an intestinal parasite) can reach levels called hyperinfections or superinfections in the host that result in increased physiologic stress or illness and death.
- As part of a program to control rhabditiform nematode infections, fecal parasite examination is important in quarantine disease screening programs. Methods for fecal parasite screening are given in Section 7.7.
- Control methods for rhabditiform parasites including prophylactic treatment with anthelmintic medication and institution of hygiene and cleaning practices that reduce exposure to pathogenic parasites are discussed in Section 8.5.

The goal of fecal parasite screening is determined in advance for each new group of animals entering quarantine. In the design of quarantine programs there is sometimes discord between philosophies that:

- Seek to control or reduce parasite loads to “normal” levels that might be observed in wild amphibian populations
- OR**
- Seek to eliminate all evidence of internal parasite infection before animals are released from quarantine.

Resolving these differences requires good communication between veterinary and animal husbandry staff. Important considerations when deciding on which approach to use for parasite control include:

- Elimination of all internal parasites is frequently impossible or is not desirable. The rhabditiform nematodes are notoriously difficult to completely eliminate even with anthelmintic treatment. Instead of protracted anthelmintic treatment courses, it can be better to emphasize reduction of parasite loads through combinations of treatment and maintenance of good enclosure hygiene.
- For populations of animals in survival assurance colonies and destined to be returned to the wild, it may be desirable to maintain low burdens of parasites known to be endemic in the wild population. This avoids concern about loss of acquired natural resistance to these organisms that may be needed for success after release (Lyles and Dobson, 1993; Cunningham 1996).
- A balance must be established between a need to control or eliminate parasites and the husbandry requirements of sensitive amphibian species. Sensitive animals will not tolerate prolonged housing under quarantine conditions and repeated administration of medication (handling, etc.). Acceptance of low parasite burdens in these animals is preferable to extended treatment. Veterinary and animal husbandry staff should conduct a cost/benefit analysis prior to parasite treatments.
- It is important to differentiate between potentially pathogenic parasites (e.g., rhabditiform nematodes) and commensal organisms found in amphibian intestinal tracts. Ciliate, opalinid and flagellate protozoa are common commensal organisms that usually do not require treatment.

The spectrum of parasites found in amphibian fecal specimens are discussed in Section 7.7.

Fecal parasite examination in quarantine

Fecal parasite screening is performed in 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.

- If the goal is to eliminate or reduce parasite burden to a predetermined level, screening is performed prior to, and after, all parasite treatments, and repeatedly until the desired level is reached.
- Increased frequency of parasite screening as an adjunct to parasite treatment is suggested if: 1) initial parasite screening shows high parasite numbers; 2) if many thin or sick animals are present within the quarantine group; or 3) if animals with heavy parasite burdens are identified on necropsy examination.

Fecal samples selected for parasite screening should be as fresh as possible and refrigerated if not immediately processed for examination. The specifics of the laboratory techniques for parasite examination and photomicrographs of commonly observed fecal parasite ova are found in Section 7.7.

Interpretation of Fecal Parasite Screening

The most important findings on fecal parasite screening are the rhabditiform nematodes. These include the amphibian lungworm, *Rhabdias* sp., and the intestinal parasites, *Strongyloides* spp. These parasites have a rapid direct life cycle that lead to superinfections if the parasite load is not mitigated. Rhabditiform nematodes have been identified as a important cause of morbidity and mortality in animals recently brought into captivity for use in survival assurance colonies (Lee et al., 2006; Pessier, 2008; Gagliardo et al., 2008).

Amphibians that have evidence of rhabditiform-type nematode infections on fecal parasite examination should be treated in quarantine. In addition to treatment with anthelmintics careful attention is paid to enclosure hygiene (see Section 8.4.)

- As noted earlier, complete elimination of rhabditiform nematode infections is difficult or impossible. It is more realistic to reduce parasite numbers and avoid introduction of heavily infected animals into established captive amphibian populations.
- Use of a qualitative scoring system for numbers of parasite eggs may be helpful in determining if parasite levels have been reduced prior to release from quarantine. Prior to release from quarantine animals should have a low number of nematode larvae or larvated nematode eggs (e.g., less than 1 egg or nematode larva per 2–3 low magnification microscopic fields on fecal wet mount examination)

6.10 TESTING AND TREATMENT FOR THE AMPHIBIAN CHYTRID FUNGUS

The amphibian chytrid fungus (*Batrachochytrium dendrobatidis* or “Bd”) is an important cause of worldwide amphibian population declines, but is also a significant problem for captive amphibians. Although susceptible species or lifestages may succumb to chytridiomycosis during a routine quarantine period, however, other species can have low-level or “subclinical” infections with Bd that are not apparent on clinical examination or in routine necropsy procedures. Subclinically infected animals can serve as a source of infection for susceptible species and attempts should be made to detect and/or treat these animals before they are introduced to zoo collections or survival assurance colonies.

Situations or species that may be of particular concern for introduction of Bd to an amphibian collection include:

- Animals recently collected from the wild.
- Animals acquired from other captive collections or commercial dealerships.
- Species that are known to commonly develop subclinical Bd infections. Examples include American bullfrogs (*Lithobates catesbeianus*), leopard frogs (*Lithobates pipiens*), eastern newts (*Notophthalmus viridescens*), tiger salamanders (*Ambystoma tigrinum*), Axolotls (*Ambystoma mexicanum*),

Hellbender salamanders (*Cryptobranchus* spp.), African clawed frogs (*Xenopus laevis*), and adult dendrobatids.

- Special care should be given when considering release of tadpoles from quarantine, because tadpoles subclinically harbor Bd in their keratinized mouthparts—not developing clinical disease until metamorphosis is completed. Infections in tadpoles, in general, may be difficult to diagnose because of the low numbers of pathogens sequestered in the mouthparts (but see Hyatt et al., 2007). Reliable antifungal treatment methods are not as widely available for tadpoles.

These examples are not all-inclusive and are not solely relied upon to determine or mitigate risk. Measures that can be applied to detecting Bd or mitigating risk of Bd infection in quarantined amphibians are:

- **Routine necropsy and histopathology of all animals that die.** Histopathology of the skin will detect characteristic lesions and Bd organisms if the animal died of chytridiomycosis (see Section 7.3 for details on diagnostic methods for Bd). Histopathology will not reliably detect animals that are subclinical carriers of Bd infection.
- **Polymerase chain reaction (PCR) testing of skin swabs for Bd DNA.**
- **Prophylactic antifungal treatment** of amphibians entering zoo collections or survival assurance colonies.
- **Holding animals at temperatures between 17 and 23°C** (if tolerated by the species) during quarantine to maximize the potential to express clinical chytridiomycosis (Young et al., 2007).

PCR testing and prophylactic antifungal treatment are the methods best suited to addressing concerns about subclinical carriers of Bd and are discussed in more detail below.

PCR Testing for Bd in Quarantine

PCR-based testing of skin swabs for Bd is available through both research and commercial laboratories and is strongly recommended as a part of routine quarantine procedures for amphibians entering zoo collections or survival assurance colonies. Details on methods of collecting samples for Bd PCR and interpreting the results are available in Section 7.2.

The PCR tests for Bd are very sensitive and detect very small numbers of zoospores (the infective stages of Bd). Despite this, false-negative test results do occur, especially with very low-level subclinical infections.

- An experimental infection trial demonstrated that a total of 3 skin swabs obtained at different times over a 14 day period will increase the likelihood that all animals infected with Bd are detected (Hyatt et al., 2007).

Multiple PCR tests may not be possible from a financial or logistical standpoint for many programs that maintain captive amphibians. Therefore a decision on the number of times to test each animal (or groups of animals) will depend on a cost-benefit analysis that includes a risk assessment for each group of animals that enters quarantine and the resources available to the institution. If animals come from a population known to be free of Bd infection (see Section 8.3) specific testing for the amphibian chytrid fungus may not be necessary. Otherwise, general recommendations are as follows:

- **Test new animals at least once during quarantine.** This approach is best if animals are coming from animal collections with a known health history and no recently identified (within 1 year) cases of chytridiomycosis. Recognize that a single test may not detect all animals subclinically infected with Bd.
- **If animals come from higher-risk situations, test at least twice and possibly three times during quarantine.** The first test should be on arrival and the subsequent tests taken at approximately equally spaced time intervals over a two-week period. Examples of higher-risk situations include: 1) animals obtained from collections with an uncertain health history or from dealerships that do not have Bd-free animal colonies; 2) animals coming from collections with recently identified cases of chytridiomycosis; 3) animals coming into captivity from the wild; and 4) instances of very valuable survival assurance colonies where the risk (however small) of introducing Bd is considered to be unacceptable.
- **Pooling of Test Samples.** In quarantine situations with large groups of animals from the same location or enclosure need to be screened for Bd infection, it may be possible to reduce the overall costs of PCR testing by obtaining swabs from individual animals, but requesting that the diagnostic lab pool those swabs into a single PCR test. Disadvantages of sample pooling are discussed in Section 7.3
- **If PCR testing for Bd is unavailable or cannot be performed,** consideration can be given to prophylactic treatment of incoming animals with antifungal medications (see Prophylactic Treatment below).

Interpretation of Test Results

Details on interpretation of positive and negative Bd PCR results can be found in Section 7.3

For purposes of making decisions on the release of animals from quarantine, the following points are considered:

- If PCR-positive animals are identified at any point during quarantine testing they should be treated with antifungal medication (see Prophylactic Treatment

below). Recognize that treatment failures do occur and be prepared to re-test animals by PCR prior to consideration of their release from quarantine.

- If some, but not all, animals from a group housed together in a quarantine room are found to be PCR-positive (or if deaths within the group are confirmed to be due to chytridiomycosis at necropsy), then the ENTIRE group is considered infected with Bd regardless of individual animal test results. The entire group should be treated with antifungal medication and re-tested prior to release from quarantine.

In either case, the length of the quarantine period will need to be extended if Bd is identified in a group of quarantined animals in order to attempt to clear infection and accommodate the time needed for follow-up testing.

Prophylactic Antifungal Treatment

Prophylactic treatment of amphibians in quarantine with an antifungal medication such as itraconazole is used either as an alternative to routine PCR testing or as an adjunct to PCR testing. Details on antifungal treatment are available in Section 8.1. Routine prophylactic treatment for Bd is recommended only in the following situations:

- **The source animals are infected or are highly likely to be infected with Bd.** An example of such a situation is animals rescued from the wild and brought into survival assurance colonies because of a known or suspected outbreak of chytridiomycosis (Gagliardo et al., 2008).
- **PCR testing is unavailable** for a variety of reasons such as location (rural or developing country) or financial considerations.
- **Necropsy or PCR testing has identified Bd infection** within a group of animals housed together in a quarantine room. In these situations the entire group should be considered infected with Bd and treated.

Potential disadvantages of routine prophylactic treatment that need to be considered when planning the quarantine process are:

- **Treatment with antifungal medication is not always well tolerated** and may be a significant additional source of stress for animals that are adapting to a new captive environment.
- **Antifungal treatment is not 100% effective.** Occasionally, two or more treatment cycles are required to completely eliminate Bd infection from a group of animals. In these instances, a single treatment cycle may give a false sense of security and result in Bd infected animals being introduced to a collection.

6.11 TESTING FOR *RANAVIRUS* INFECTION

Infections with ranaviruses are an important cause of mass mortality events in wild amphibian populations, but there are only occasional reports of *Ranavirus* infection in captive amphibians (Miller et al., 2008; Pasmans et al., 2008). Reported outbreaks in captive animals been small or limited to a single species.

- It is unknown if there is a low prevalence of infection in captive animals; if infection in captive animals have gone unrecognized because the clinical and pathologic findings overlap with those of other infectious diseases in amphibians (e.g., “red leg syndrome”); or if *Ranavirus* infections that have occurred in captive populations are of relatively low virulence.
- The ranaviruses are a large group of related viruses each with unique biological behavior. This variability in behavior and gaps in knowledge of *Ranavirus* biology interferes with design of well-designed control and testing programs for captive collections.
- Subclinical infections with ranaviruses have been documented (Brunner et al., 2004; Robert et al., 2007), but it is unclear if subclinical infections are transient or persistent for long periods of time. It is possible that subclinically infected animals could serve as a source of infection for uninfected animals, however, this has not yet been documented for captive amphibian collections.
- The major concerns about ranaviruses for captive amphibian programs are the possibility of a significant mortality event in a valuable species (or throughout an amphibian collection) or that subclinically infected captive populations might serve as a vector for movement of novel ranaviruses into naïve wild populations.

Testing for Ranaviruses in Quarantine

Routine testing of living healthy animals for *Ranavirus* infection in quarantine is not recommended. Unlike testing for the amphibian chytrid fungus, the available PCR tests for ranaviruses are not validated to reliably screen living amphibians for subclinical infection.

- The lack of a reliable test means that there are significant disadvantages to routine testing of living healthy animals. These include significant costs of testing (with unclear benefits), stress on quarantine animals resulting from sample collection, and the possibility of developing a false sense of security from negative test results.
- Be aware that some laboratories are willing to perform testing on these samples regardless of the ability to accurately interpret the results.
- As advancements are made in the understanding of *Ranavirus* biology and diagnostic testing, recommendations are likely to change.

If testing of animals in quarantine is desired. The best samples to submit are tissue samples collected at necropsy of animals that die in quarantine and/or tissues

collected from animals in the quarantine group that are culled specifically for disease screening purposes. Details on diagnostic testing for ranaviruses is available in Section 7.4

At this time, the most important consideration in a quarantine situation is to avoid introducing animals into collections that are clinically ill with an active and clinically significant *Ranavirus* infection. Approaches that can minimize this risk include:

- **Review of the health history from the source population of animals.** If there have been recent (6 months or less) deaths or illness in the source population that have been confirmed as being due to *Ranavirus* infection, the risk of introducing sick animals is higher. The health history of the source population should also be reviewed for clinical signs or necropsy findings that could be suggestive of *Ranavirus* infection. These include:
 - hemorrhage in multiple tissues, especially skin (“red leg syndrome”) and gastrointestinal tract.
 - necrosis in liver, kidney, gastrointestinal tract or hematopoietic tissue.
 - proliferative or ulcerative skin lesions.

It should be acknowledged that these clinical signs and findings are both non-specific and common in captive amphibians. If suspicious clinical signs or lesions are identified, attempts can be made to confirm or rule out a *Ranavirus* infection (see below).

- Necropsy and histopathology surveillance (as well as clinical observations) to detect outbreaks of disease in quarantine animals that have potential features of *Ranavirus* infection (see above).
- If outbreaks of disease and/or necropsy findings are suggestive of possible *Ranavirus* infection, use PCR testing to confirm or to rule out infection. The best samples for PCR are liver, kidney and skin (if skin lesions are present) These samples are collected at necropsy and stored frozen. If frozen tissues are not available it may be possible to perform PCR from the tissues embedded in paraffin for histopathology. Samples can also be attempted from clinically ill living animals such as cloacal or pharyngeal swabs, tissue biopsy or blood. Details on sample collection techniques for *Ranavirus* PCR are available in Section 7.4.

Interpretation of Diagnostic Tests for *Ranavirus*

If clinical illness or deaths attributable to *Ranavirus* infection are identified in a group of quarantined animals, the quarantine period is extended indefinitely. A decision about the disposition of animals remaining after an outbreak will depend on consideration of multiple factors such as:

- **The importance of the infected or exposed animals to the captive population and species recovery efforts.** If disease attributed to *Ranavirus* infection is identified during quarantine in a common species entering a collection for display purposes euthanasia of remaining animals may be considered. If disease attributed to a *Ranavirus* infection occurs in a valuable group of animals (e.g., an endangered species and the individual animals are deemed critical to the recovery program or survival assurance population), additional efforts can be considered with the hope that animals will eventually clear any remaining subclinical infection. Animals in this situation are managed as a separate population from the primary survival assurance population until long-term disease surveillance demonstrates that they are truly free of *Ranavirus* infection. See Section 8.2.
- **Occurrence and duration of subclinical *Ranavirus* infection.** The potential duration of subclinical *Ranavirus* infection is unpredictable and probably dependant on multiple factors such as the host species, the species of *Ranavirus*, and other unidentified influences. Experimentally infected *Xenopus laevis* appeared to clear infection with Frog Virus 3 (a type of ranavirus) in as little as 20 days, however, in contrast a group of tiger salamanders subclinically infected with *Ambystoma tigrinum* virus had evidence of infection for at least six months (Brunner et al., 2004; Robert et al., 2007). If subclinical infections can be cleared naturally, an extended quarantine period may be all that is needed for safe release of animals from quarantine. However as mentioned earlier, detection of subclinical infection is problematic and any decision to release animals from quarantine would require careful risk assessment.
- **The results of follow-up disease surveillance during an extended quarantine period.** Testing that might be helpful includes:
 - For larger groups of animals, consideration is given to culling (euthanasia) of animals from the quarantine group for diagnostic testing. Culling in this situation is most useful for detecting persistent subclinical infections if it is performed several weeks after clinical illness or deaths due to *Ranavirus* infection have stopped.
 - Necropsy and histopathology of all animals that die or are that are culled for disease surveillance to look for characteristic lesions of *Ranavirus* infection.
 - PCR of liver and kidney for *Ranavirus* collected at the time of necropsy from all animals that die or animals that are culled for disease surveillance.
 - PCR from living animals (an exception to the previous recommendation to not perform PCR on healthy living animals).
- **Presence or absence of pre-existing *Ranavirus* infections in the captive population and wild population.** This requires comprehensive health surveys of the relevant wild and captive populations to establish baseline information. This consideration is most relevant to survival assurance populations of animals in permanent isolation from a general amphibian collection. For example, in a

situation where the wild populations (= original source of the founder animals for the colony) are known to have endemic *Ranavirus* infections occurring naturally within the range of the species, then a decision may be made to manage the *Ranavirus* infections in the isolated captive population. The decision to do this is complicated and should be decided as part of an overall management plan for the species under consideration. Factors that need to be considered include: 1) verification that the precise species of *Ranavirus* present in the wild and captive population are the same (this process is more complicated than simple PCR testing, see Section 7.4; and 2) determination of the likelihood that the ranaviruses present in the wild and captive population are truly endemic viruses and not exotic viruses that have been introduced to the wild population.

Confidence in the results and value of disease surveillance for ranaviruses increases with the number and quality of samples available over time for diagnostic testing.

6.12 CLINICAL PATHOLOGY (HEMATOLOGY AND CLINICAL BIOCHEMISTRY)

General clinical pathology data can be collected during quarantine to augment our understanding of the normal physiology and ecology of different amphibian species.

- Interpretation of hematology and clinical biochemistry in amphibians is in its infancy, and there is limited immediate diagnostic significance to any one test.
- Information obtained might be useful in the future to enhance disease detection and improve overall conservation efforts.
- If clinical pathology data is collected, efforts should be made to carefully record the laboratory methods used to obtain data as well as the numerical data itself.

If practical, based on factors such as animal size, finances and the availability of a suitable testing laboratory, a full hematologic and biochemistry panel can be performed on each animal during quarantine.

- Blood collection is usually performed during a quarantine physical examination.
- It is not recommended to collect blood from animals weighing less than 50 grams due to safety concerns. In group situations, a representative number of animals (20–30% of the group) can be sampled.
- Until our understanding of normal amphibian hematology and clinical biochemistry improves, interpretation of data should be circumspect and rely on clinician experience as well as published suggestions (Wright and Whitaker, 2001). A variety of factors including hydration status, seasonality and sex can influence the outcome of hematological and biochemical parameters to a greater degree than that observed in other vertebrate animals.
- There are no current recommendations for hematological/biochemical criteria that should be used as an exclusionary tool to prevent an animal's release from

quarantine. One can consider elevations or decreases in key assays like albumin, white blood cell counts, etc, to base an intuitive guess to prevent quarantine release in a suspect animal. However, given the number of normal animals in the reference range for a species is currently so low as to make all statistical interpretation clinically useless and ill advised.

Details on sample collection and analysis of clinical pathology information is available in Section 7.8

6.13 OTHER INFECTIOUS DISEASES ENCOUNTERED IN QUARANTINE

Aerobic Bacteria (e.g., *Aeromonas hydrophila*)

Infections with a variety of Gram-negative bacteria, including *Aeromonas hydrophila* or *Pseudomonas* spp., have been implicated as the cause of bacterial dermatosepticemia (red leg syndrome) in captive amphibians (Taylor et al., 2001).

- There is little or no benefit in performing routine bacterial cultures on apparently healthy amphibians in quarantine. Bacteria such as *Aeromonas* are common inhabitants of aquatic environments and are frequently isolated from healthy animals.
- Bacterial cultures are helpful for clinical management (e.g., antibiotic susceptibility patterns) of sick animals in quarantine.
- Control of bacterial infections in quarantined amphibians focuses on reducing factors that often contribute to their development such as stress of recent shipment, overcrowding, or poor water quality (e.g., with high organic loads).

Enteric Bacteria (e.g., *Salmonella*)

Like reptiles, amphibians sometimes carry enteric bacteria such as *Salmonella* spp. that have zoonotic potential (Taylor et al., 2001; Centers for Disease Control and Prevention, 2010).

- Routine screening for enteric pathogens and treatment of infected animals is not suggested. Instead, hygiene practices should be in place to reduce the likelihood that human caretakers will become infected with enteric bacteria of amphibian origin.
- Guidelines for hygiene practices are available online:
 - Compendium of Measures to Prevent Disease Associated with Animals in Public Settings 2009—Centers for Disease Control and Prevention.
www.cdc.gov/mmwr/preview/mmwrhtml/rr5805a1.htm
 - Salmonella Bacteria and Reptiles—Association of Reptile and Amphibian Veterinarians.
www.arav.org/ECOMARAV//timssnet/arav_publications/SalmonellaOwner.pdf

Chlamydophila

Infections with *Chlamydophila psittacii*, *Chlamydophila pneumoniae*, and other chlamydial-type organisms are increasingly being recognized in both captive and free-ranging amphibians (Corsaro and Venditti, 2004; Blumer et al., 2007).

- Chlamydiae can have a variety of presentations including unexpected death or a resemblance to “red leg syndrome.”
- Lesions that may be observed on histopathology are necrosis and non-suppurative inflammation in tissues such as the lung, kidney, liver and heart. In most cases, intracellular fine granular basophilic cytoplasmic inclusions may be observed by histopathology in affected tissues.
- Confirmation of the diagnosis is by immunohistochemistry using Chlamydiaceae family-specific anti-LPS monoclonal antibodies or by polymerase chain reaction (PCR) for chlamydial 16sRNA and ompA genes. Routine testing of healthy animals in quarantine is not recommended.

If chlamydiae is detected the quarantine period should be extended for the remaining animals in the group to monitor for the development of clinical signs of disease. Treatment of remaining animals with tetracycline group antibiotics may be considered (Taylor et al., 2001). PCR testing may be attempted using cloacal swabs, but these tests have not been validated for this purpose and routine screening of healthy animals is not recommended.

Mycobacteriosis

Infection with acid-fast bacteria in the genus *Mycobacterium* including *M. fortuitum*, *M. marinum*, *M. chelonae*, and *M. xenopi*, among others are a common problem in captive amphibian collections.

- These organisms are ubiquitous inhabitants of aquatic environments (Primm et al, 2004).
- These mycobacteria are usually opportunistic pathogens occurring secondary to factors such as skin injury, poor husbandry, or stress.
- Epidemiologic studies of amphibian mycobacteriosis are required, the scenario in captive programs may be similar to that documented for zoo birds where the original source of infection is an environmental reservoir rather than animal-to-animal transmission of a particular organism (Schrenzel et al., 2008).
- Very heavily infected animals might still act as a source of infection for other animals in a group because they can shed large numbers of organisms that exceed the “normal” levels of exposure to these bacteria that occurs in aquatic habitats.
- The goal in quarantine situations is not to detect every animal that has an underlying mycobacterial infection, but instead to avoid introduction of animals

with overt clinically significant mycobacteriosis (that have the highest potential for shedding high organism numbers) to an amphibian collection.

Clinical signs of mycobacteriosis are most often related to the skin and include cutaneous ulcers, abscesses, skin nodules and swollen extremities (cellulitis) that do not resolve with supportive care or antibiotic or antifungal therapy.

- Dissemination to visceral organs such as the liver and kidney occurs frequently. In disseminated infections, non-specific signs such as weight loss, despite good appetite, may be observed.
- Infections can present as outbreaks in a population, but often appear as sporadic events limited to a single animal.

Diagnosis of mycobacteriosis is problematic in animals without obvious skin lesions.

- Screening acid-fast stained slide preparations of feces, oropharyngeal mucus or cutaneous slime layers are not predictive of disease in outwardly healthy animals and are not recommended.
- Similarly, PCR tests are available for the environmental mycobacteria, but the utility for reliable diagnosis from samples such as cloacal or skin swabs in outwardly healthy animals is unknown (strong potential for both false-negative and false-positive results).
- If skin lesions are observed, cytologic examination of touch preparations or aspirates by acid-fast staining can provide a rapid diagnosis (Pessier 2007). Biopsy and histopathology of skin lesions with acid-fast staining are also diagnostic.

For managing mycobacteriosis in quarantine:

- Review the health history of the source population. If there have been recent (within 3–6 months) cases of mycobacteriosis, extend the quarantine period (90 days) to observe incoming animals for skin lesions or evidence of chronic weight loss or other non-specific signs of poor health.
- If animals with mycobacteriosis are identified in a quarantine group:
 - Place the remaining animals from the group into an extended quarantine period (90 days) to observe for skin lesions or evidence of chronic weight loss or other non-specific signs of poor health. Remove sick animals that show evidence of infection either to a separate isolated enclosure or consider culling and euthanasia. This option may be most desirable for animals that are very valuable such as an endangered species critical for breeding in a survival assurance colony.

- If animals are less valuable or an extended quarantine period is not possible, consideration can be given to euthanasia of directly exposed (same cage) animals with or without external signs of disease.
- Do not release from quarantine animals with mycobacterial skin lesions or other potential signs of mycobacteriosis. Treatment of amphibian mycobacteriosis has generally not been successful and euthanasia of animals with clinical signs of disease is recommended. Treatment can be considered as a salvage measure for very valuable animals.
- Animals from a group with confirmed cases of mycobacteriosis can be candidates for release from quarantine if after 90 days of observation, they do not show skin lesions or other clinical signs that could be due to mycobacterial infection.
 - This does not guarantee that animals are free of mycobacterial infection, but reduces the risk of introducing animals that are shedding high numbers of organisms into the zoo collection or survival assurance colony.
 - Consider initially introducing these animals to situations where there are only small numbers of other animals in direct contact (same cage).
- Finally, infection with the environmental mycobacteria has been considered a potential zoonosis.
 - The overall risk of animal to human transmission of these organisms is low and measures that can be helpful in avoiding human infection include: 1) wearing gloves during servicing of enclosures or during animal handling to minimize the potential for inoculation of organisms into skin wounds; and 2) avoiding aerosols of water or environmental materials (such as contents of biological filtration systems). Use of facemasks or face shields may be helpful in this regard.

Fungal and Water Mold Infections

A huge variety of cutaneous and systemic fungal infections occur in captive amphibians. The most common infections are mycotic dermatopathies caused by organisms such as *Basidiobolus ranarum*, *Mucor sp.*, *Fusarium sp.* and the water molds such as *Saprolegnia* (Taylor, 2001; Pessier, 2003).

- Almost all the miscellaneous fungi that affect amphibians are ubiquitous in the environment and usually occur as secondary infections (rather than acting as primary pathogens).
- Complete elimination of these fungi from enclosures is not practical. Emphasis is placed on husbandry practices to reduce animal stress and provide an environment free of excessive wastes (food, feces, decaying material) that can provide the substrates for fungal overgrowth and improving water quality (e.g., ammonia exposure). All of these environmental factors contribute to the development of fungal infections.

Diagnosis of fungal infections in quarantine is through direct observation and diagnostic investigation of skin lesions (i.e., skin scrape for cytology or a skin biopsy) or histopathology of deceased animals.

- Cultures from the skin and affected visceral organs can help determine the fungal species infecting the animal. Culture interpretation can be problematic because of uncertainty if the cultured fungus is simply in the environment or actually causing disease in a particular animal.
- Treatment is twofold: 1) treat the affected animals through topical and/or parenteral medications such as itraconazole; and 2) improve husbandry practices to improve water quality, reduce fungal exposure and animal stress.

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Chapter 7

DIAGNOSTIC TESTING

7.0 INTRODUCTION

Control of disease problems in amphibian survival assurance colonies and reintroduction efforts depends on disease surveillance programs, accurate identification of these problems by laboratory methods and appropriate interpretation of laboratory results.

Some important aspects of disease testing and surveillance include:

- Principles of sampling populations for infectious agents (e.g., determining the number of animals that must be sampled to be confident that a population is free of an infectious agent).
- Evaluation and identification of the laboratories that can perform diagnostic testing on amphibian samples.
- Selection of the most appropriate diagnostic test for the infectious agent of interest and for the situation or context that the test will be used.
- Collection of the best sample type for the test that will be performed.
- Proper interpretation of test results (especially important for molecular diagnostic testing or “PCR”).
- Use of the necropsy (postmortem examination) as an important tool for identifying and managing disease problems in amphibian colonies (both infectious and non-infectious diseases) and for performing disease risk assessments in amphibian reintroduction programs.

This chapter aims to provide an overview of these topics and includes:

- Discussion of methods, diagnostic sample collection, and test interpretation for commonly used laboratory methods for control of infectious diseases in amphibian survival assurance colonies.
- There is an emphasis on frequently used molecular diagnostic tests for important pathogens (e.g., amphibian chytrid fungus and ranaviral infections) and screening for fecal parasites.
- Necropsy examination is discussed in detail in Chapter 9.
- A list of laboratories that accept amphibian samples.

It is hoped that this compiled information will be of use both by personnel in range country locations that do not have readily available veterinary support as well as veterinarians that are working to develop amphibian health protocols.

7.1 DISEASE SURVEILLANCE

Disease surveillance is the practice of looking for disease with the intent of controlling it if needed. In conservation programs, disease surveillance of both *ex situ* (captive) and *in situ* (wild populations) is necessary to detect disease problems that can be threatening to the sustainability of survival assurance populations and the success of reintroduction programs. Indeed, because infectious disease has played such an important role in many amphibian population declines, control of disease problems that occur *in situ* populations is necessary for the success of *ex situ* conservation efforts that involve reintroduction of animals to the wild. Disease surveillance is necessary to:

- Detect and mitigate disease problems before they result in mortality events that can have negative effects on populations.
- Rapidly determine the cause of mortality in a population with the aim of limiting the impacts of disease outbreaks.
- Limit the potential for spread of infectious diseases. This is especially important when amphibians are moved from the wild to captivity, between captive facilities and from captivity to the wild.
- Gather information needed for disease risk assessments when planning amphibian quarantine or reintroduction.
- Detection of potential zoonotic diseases to maximize the health and safety of conservation workers.

Specific recommendations for when disease surveillance is necessary are listed in earlier sections of this manual dealing with risk assessment, quarantine and biosecurity. Disease surveillance needs to be tailored to each amphibian species, and depends on factors such as susceptibility to each pathogen of interest, presence of the pathogen, long-term conservation goals for the species under consideration and practical consideration of the resources available. With this in mind flexibility and prioritization of surveillance programs are important.

How is Disease Surveillance Conducted?

Disease surveillance is designed to monitor a population for novel pathogens as well as determine the disease status of a population, disease burden (prevalence) or rate of disease spread (incidence) within the population, or other characteristics such as disease-related morbidity or mortality. For example, does the population have disease or not? What proportion of the population has disease? Is there an increase in the number of new infections? Surveillance can be specifically targeted to known infectious agents of significance (e.g., amphibian chytrid fungus) or can be designed to detect a variety of different potential disease problems.

- The first step is to collect a history of the population of interest as well as cohort species that may occur in the wild or within a captive facility. Information that may

be valuable includes: population size, age structure, original source (e.g., bred at same institution, wild caught), source of food items, dietary history, history of body condition, etc.

- Depending on the basic question at hand, high-risk groups for disease within the population can be targeted for testing such as sick or dead animals (see Chapter 9) in order to increase the probability of detecting disease.
- It is usually more cost efficient to test a portion of individuals in the population rather than test the whole group or population. There are several population sampling methods but we recommend random sampling methods when feasible and appropriate to avoid potential sampling bias (see below for additional sampling methods).
- For determining the disease status of populations appropriate sample sizes must be obtained.

Defining the Population of Interest

The population of interest for disease investigations will be different depending on the reason for sampling or the research question of interest. When defining the sampling population, consideration should be given to characteristics of the source population(s) including, but not limited to:

- Specified time period for surveillance.
- What is the species of interest (e.g., a single species; all species from a specified region or habitat).
- Is the study focused on a specified region or facility or enclosure.
- Is the study focused on sick animals; animals presented for necropsy; animals tested for a specific infectious disease.
- Is the study focused on a specific disease or species where you should consider important life-history characteristics to better define the source and sampling populations? For example, does the disease only affect adults (in which case the sampling population may need to target adults only)?

Sampling the Population

The methods by which sampling of populations is conducted is important because differences in sampling methodology can:

- Bias the results you obtain and affect the perceived prevalence or presence of disease in the population.
- Affect how well the diagnostic test performs.
- Affect how well the diagnostic tests perform, thereby impacting the investigator's ability to answer their research question.

Several different disease-sampling methods in animal populations have been developed and the choice of sampling method should be based on the specific purpose of sampling, the

study design, and the ease of acquiring samples. Probability (based on equalizing the opportunity that a particular subject is selected) and non-probability (based on convenience sampling or purposive sampling of high-risk groups) methods utilizing simple and complex designs (e.g., stratified or cluster sampling) have been developed and can be reviewed in standard veterinary epidemiology texts (Thrusfield, 2007).

When sampling amphibian populations for disease, it is important to keep in mind how individuals are chosen to be tested for specific pathogens and how it can affect the interpretive outcome. To estimate disease prevalence in a population samples from a representative subset of the source population are needed.

- If not sampling the entire population, these subjects should be chosen using systematic random sampling methodology when possible. For example, if 30 out of 100 amphibians are going to be sampled, the sampled amphibians should be randomly selected and represent the same enclosures or similar enclosure use as the rest of the population.
- Keep in mind behavioral differences in amphibians that may affect the interpretation of disease detection or prevalence. For example, individuals that are easier to catch may be disproportionately infected with a pathogen (i.e., the animals easiest to catch are the sick ones) and, accordingly, bias your infection prevalence estimates higher than they really are. Similarly, individuals that use the enclosure space in a particular way may also bias the outcome if behavior is influenced by infection.

Determination of Sample Size

Testing for pathogens can be targeted towards individual animals to assess individual health or whole populations to determine the health or infection status of the population. When testing populations, considerations need to be given to sample size in order to:

- Ensure that enough individuals are selected to answer a test question of interest.
- Determine the precision for which prevalence and other statistical estimates can be made.
- Avoid unnecessary, over-sampling when resources are limited; this can be costly and time-consuming and does not always provide additional information on population-level measurements of disease.

The sample size required to determine if an infectious agent is present in a population or what the infection prevalence in a population is usually requires:

- Knowledge or estimates of the population size from which the samples will be obtained.
 - Generally, when the population size is large, a larger number of individuals in the population (but a smaller proportion of the total population) will need

to be sampled to estimate prevalence or detect the presence of a disease, as compared to sampling in a small population.

Generally, when the population size is small, a larger proportion of the population (but smaller total number) will need to be sampled to estimate prevalence or detect disease, as compared to sampling in a large population. If the population is really small, nearly all individuals may need to be sampled to try and answer test questions of interest.

- The expected prevalence (or incidence) of infection in the population (i.e., the proportion of the population with infection). Often the expected prevalence that is used in a sample size calculation will be a guess. A good guessing strategy should be designed to maximize the needed sample size to answer the question of interest, without unnecessary over-sampling.
 - When calculating the sample size to *detect the presence of disease* in a population, use the minimum expected prevalence (or incidence). In these calculations, a smaller expected prevalence yields a larger sample size. For example, if you estimate the prevalence to be between 2% and 10%, calculating a sample size that corresponds to the smaller prevalence (2%) will yield a larger sample size and is the better prevalence estimate to use.
 - When calculating the sample size to estimate disease prevalence (or incidence), it will take a larger sample size to estimate the prevalence at 50% at a specified precision than at 0% or 100%. For example, if you think the prevalence is between 10% and 20%, choosing the 20% prevalence estimate for sample size calculations will yield a larger sample size. Similarly, if you think the prevalence is between 80% and 90%, choosing the 80% prevalence value for sample size calculations will yield a larger sample size. Since the maximum sample size for estimating prevalence is always at 50%, prevalence estimates closer to this figure will always yield a larger sample size, and may be the more conservative prevalence estimates to use.
- The desired level of significance (statistical precision) in finding at least one infected animal, or determining prevalence. This is usually set at a 95% confidence interval (or $\alpha = 0.05$).
 - As previously stated, in general, if a population is large, then more individuals will be needed to identify a single infected animal. This is true up to a certain point when the number needed plateaus and will depend on the desired statistical precision.
 - For very small populations, all individuals may need to be sampled, and yet the actual status of disease in the population may not be determined to the desired degree of statistical precision.
- Knowledge of the accuracy of the test. This is comprised of the test sensitivity (ability to classify all infected animals as positive for the infectious agents based upon the test method) and the test specificity (the ability to classify all non-infected animals as negative for the infectious agent based upon the test method).
 - Many sample size calculations do not incorporate test accuracy assessments and assume that the diagnostic test is always 100% accurate. While

appropriate in some situations, if diagnostic tests do not perform well for any reason, such calculations may bias your sample size estimate to be smaller than what is needed to address the study questions.

- Formulas for incorporating imperfect tests into sample size calculations are complex, but have been incorporated into Freecalc software (see Thrusfield, 2007, for discussion of Freecalc’s treatment of sample size calculations with imperfect tests). It may be useful to include assessments of test sensitivity and specificity with sample size calculations when possible, although these parameters often are unknown.
- If adjustments for imperfect diagnostic tests are not included, then investigators should bear in mind how test accuracy may affect the survey interpretation and outcome and determine whether adjusting sampling methods (e.g., sampling more animals) would be warranted.
- Many of the parameters needed for these calculations are unknown for amphibian diseases.

Sample size calculations can be specific to a study design, expected prevalence, estimated population sizes, questions of interest, etc. Calculations can be complicated and may require input from an epidemiologist or a statistician. Examples of equations that can be used to calculate sample size are provided in the appendix or standard textbooks of veterinary epidemiology (see Thrusfield, 2007). Tables that may help to derive quick sample size approximations are also available in epidemiology texts (See Thrusfield, 2007) and some online calculators are available (see below); however the investigator should be cognizant of the underlying equations (and their assumptions) used in any table or calculator and assess the appropriateness for their particular study.

- AusVet Animal Health Services: <http://epitools.ausvet.com.au/>
- OpenEpi: <http://www.openepi.com/Menu/OpenEpiMenu.htm>
- EpiInfo: <http://www.cdc.gov/EpiInfo/>

7.2 EVALUATION OF LABORATORIES THAT PERFORM MOLECULAR DIAGNOSTIC TESTING (POLYMERASE CHAIN REACTION “PCR”)

Molecular diagnostic testing using polymerase chain reaction (“PCR”) based tests for infectious agents such as the amphibian chytrid fungus or ranaviruses are useful tools for the management of disease problems in captive amphibian populations and survival assurance colonies. These tests have become very common for: 1) screening animals in quarantine; 2) screening of populations (wild or captive); 3) screening prior to use of animals in reintroduction programs; and 4) for the diagnosis of sick animals.

- For many important amphibian pathogens, the protocols for performing these tests are published in the scientific literature and the equipment needed to perform the tests are widely available in university and private laboratories. As a result testing for amphibian pathogens is now available from a number of different sources.

- Laboratories offering testing vary tremendously in:
 - Experience with diagnostic testing for infectious disease agents.
 - In the ability to help you properly interpret test results.
 - Timeliness of return of results.
 - In the ability to provide reliable test results.
- Appropriate practices, test validation and quality control measures must be used in the laboratory to avoid erroneous or misleading information.
- Guidelines for proper collection and handling of samples may be different between laboratories. Good communication with the laboratory selected to process samples is essential for optimal test results. Checking references for the lab is advisable.

This section provides some questions and guidelines that can be used to evaluate and select a laboratory that will provide molecular diagnostic testing for important amphibian pathogens.

What type of test does the laboratory use? There are two major types of PCR test: conventional PCR and real-time PCR.

- If the laboratory uses conventional PCR, ask how positive results are verified. In conventional PCR, the product of a test reaction is usually visualized by agarose gel electrophoresis, which separates DNA fragments by size. Because non-pathogen DNA fragments can have a similar size as the pathogen DNA fragments, it is possible to get false-positive results if only gel electrophoresis is performed. Therefore it is important to consider whether DNA sequencing or specific hybridization are needed to verify the results of the analyses.
- Real time PCR (if well-validated in the laboratory) has some advantages over conventional PCR. These include: 1) increased test sensitivity; 2) no need to verify positive results by DNA sequencing if the Taqman real-time method is used (a specific DNA probe is included in the process); 3) the possibility to include internal controls that detect PCR inhibitors; and 4) rapid results.
- If the laboratory uses real-time PCR, ask if the test uses fluorescent dyes (Sybr green) or fluorescent reporter probes (Taqman). Taqman PCR utilizes an internal probe to increase fidelity of results and sybr green quantitative PCR relies on melting curves and therefore is less specific (possibility of false positive results).
- If the laboratory uses real-time PCR ask how many times the test is run for each sample. It may be important to run samples in triplicate due to inherent errors in real-time PCR due to small volumes, pipetting errors, and deviations in laser detection of some individual wells.
- Regardless of the type of PCR used, ask the lab what they use as negative and positive control samples. With real-time PCR, a dilution series of samples known as a titre must be run with every batch of test samples, if any sort of quantitative information can be determined.

What are the sensitivity and specificity limits of the test? In reference to PCR testing it is important to distinguish between “analytical” sensitivity and specificity and “diagnostic” sensitivity and specificity (Saah and Hoover, 1997). There will never be a test that is both 100% sensitive and 100% specific, as there always will be a tradeoff between these two parameters. A test that is highly sensitive will have a lower specificity and vice versa.

- **Analytical sensitivity** for PCR tests is the lowest concentration of pathogen DNA that can be detected by the test.
 - It is determined in the laboratory using dilutions of a known DNA standard of the pathogen of interest. The analytical sensitivity of the Taqman PCR for amphibian chytrid fungus can be as low as 0.1 genome equivalents (a single chytrid zoospore in practical terms; Boyle et al., 2004).
 - Laboratories should be able to provide the analytical sensitivity of their tests
 - Diagnostic sensitivity describes the proportion of true positives (samples that contain the DNA of the pathogen) detected by a test.
 - A sensitivity of 100% means that the test identifies all the true positive samples and that there are no false negative samples (samples that contain DNA of the pathogen, but are not detected by the test).
 - Few tests will have 100% diagnostic sensitivity, even if they have very high analytical sensitivity. This is because of variability inherent in sample collection and processing.
 - Very few laboratories will be able to provide a diagnostic sensitivity because this calculation requires correlation of test results to animals that are of known positive or negative status. Diagnostic sensitivity can also depend on factors outside of laboratory control such as the method that is used to collect samples. The diagnostic sensitivity of the Taqman PCR procedure for the amphibian chytrid fungus has been determined in one laboratory to be approximately 75% (Skerratt et al., 2008).
- **Analytical specificity** for PCR tests is the ability to exclusively identify the DNA of the pathogen of interest and not the DNA of other organisms.
 - It is usually performed by “challenging” the test with organisms closely related to the pathogen of interest.
 - For example, determining the analytic specificity of PCR for the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) requires that the test only give positive results for *Batrachochytrium* and not a panel of other chytrid fungi (Boyle et al., 2004).
 - The laboratory should be able to make a statement about the analytical specificity for the test that they offer.
- **Diagnostic specificity** is the proportion of true negatives detected by the test. A specificity of 100% means that all true negative samples were identified and that there are no false positive samples (i.e., samples that do not contain DNA of the pathogen, but are interpreted as positive).
 - Diagnostic specificity can vary for PCR tests. Because these tests have high analytical sensitivity, it is easy for samples to become contaminated with

pathogen DNA during sample collection or in the laboratory, resulting in false-positive tests. False-positive tests reduce the diagnostic specificity.

What quality controls are utilized in your test?

- The laboratory should use negative and positive controls every time the test is performed. This is absolutely necessary for credible results.
- Positive controls use known DNA from the pathogen of interest and show that the test is able to amplify this DNA and that the test is working properly.
- Negative controls do not contain DNA from the pathogen of interest and are used to detect DNA contamination in the laboratory or laboratory reagents.
- For the real-time PCR methods, laboratories should use an internal positive control (Hyatt et al., 2007). This is a separate PCR reaction of DNA not related to the pathogen of interest and is included in each sample that is analyzed. The internal positive control detects PCR inhibitors that can result in false-negative tests (decreases the diagnostic sensitivity of the test). For example, skin swabs for the amphibian chytrid fungus frequently contain PCR inhibitors and use of the internal control is essential (Hyatt et al., 2007; Skerratt et al., 2008).
- PCR inhibitors are also a problem for laboratories that perform conventional PCR. The laboratory should be asked how they approach this potential problem.
- If quantitative results are desired from real-time PCR methods it is important to ask how the quantification standards were generated and handled. It has been shown that degradation of stored standards occurs with some protocols leading to underestimation of chytrid fungal loads.

What samples types are ideal for your test and how should they be collected, stored and shipped?

- It is important to know the correct sample type for the type of test being performed. For example, testing for the amphibian chytrid fungus requires a swab or other sample from the skin, and for anurans the best sample comes from the ventral skin surfaces. Samples taken from the dorsal skin or taken from another body location such as the liver will not be valid and will provide misleading results. Similarly, a swab of the skin is usually not a good sample to detect ranaviruses which usually infect internal organs or tissues.
- The choice of sample collection materials can be important. As an example, the swabs that are used to collect samples for amphibian chytrid fungus are made of different types of materials (wood, metal or plastic handles). For the Taqman PCR method, plastic handled rayon swabs work better than wood handled swabs. The laboratory should be consulted for advice on the best materials to be used for sample collection.
- The preferred methods for sample storage will depend on the type of test performed. For example, the Taqman PCR method for the amphibian chytrid fungus

works better on air-dried swab samples, but some laboratories that perform the conventional PCR methods prefer swabs preserved in ethanol. Always ask the laboratory for advice on how to store samples after collection.

- The methods used to ship samples are very important for the results of PCR testing. As an example, swabs taken for PCR to detect the amphibian chytrid fungus may degrade if exposed to very high temperatures (Van Sluys et al., 2008) and therefore measures should be taken to keep samples cool after collection and during shipment. If PCR is to be performed on tissue samples for ranaviruses or other infectious agents, it is important that tissues remain frozen during shipment, or are preserved in high quality non-denatured 70%+ ethanol. Samples destined for any type of PCR diagnostic cannot, under any circumstances, come into contact with formalin or other types of preservative. Similarly, isopropyl alcohol cannot, under any circumstances, be used instead of ethanol.

How long will it take to obtain results?

- Laboratories that perform molecular diagnostic testing for amphibian infectious diseases have become very busy and it can sometimes take several weeks to months to get results.
- Long waits (turn-around time) may be unacceptable for some situations (e.g., diagnosis of sick animals or for testing in quarantine or prior to release of animals into the wild). In these situations it is important to communicate with the laboratory to arrange for more rapid results if possible.

Who has ownership of the samples and results?

- Communication and collaboration with the laboratory is essential regarding the ownership of samples and the results of testing. Tell the laboratory in advance if you plan on doing further research or publishing the results from the samples submitted.
- Research laboratories may be able to perform testing at low-cost if the results are incorporated into ongoing research projects or publications.
- It should be verified before testing takes place that the proper permits for collection, exportation, and/or importation have been obtained.

7.3 DIAGNOSTIC TESTING FOR THE AMPHIBIAN CHYTRID FUNGUS

(*BATRACHOCHYTRIUM DENDROBATIDIS*)

The amphibian chytrid fungus (*Batrachochytrium dendrobatidis* or “Bd”) is an important cause of worldwide amphibian population declines, but is also associated with significant mortality events in captive amphibian populations. Although susceptible species or lifestages may succumb to chytridiomycosis during the course of a routine quarantine period, it should be recognized that some species or individuals can harbor low-level or “subclinical” infections with Bd that are not apparent on clinical examination or in routine

necropsy procedures. Subclinically infected animals can serve as a source of infection for susceptible species and attempts should be made to detect and/or treat these animals before they are introduced to zoo collections or survival assurance colonies. It is important to emphasize that the OIE and Berger et al. (1998) define chytridiomycosis as infection with Bd. This is the convention with disease now as even subclinical infection may have an effect that is considered disease such as reduced reproduction or dispersal.

Selection of a Diagnostic Test

When selecting a diagnostic method it is helpful to distinguish between disease or death due to Bd infection and subclinical or sublethal infection (carrier animals that have no outward signs of infection or disease).

- Morphologic methods such as wet mounts, cytology and histopathology are useful for diagnosing animals that are sick with chytridiomycosis because large numbers of organisms are present in the skin. They are not reliable for detecting subclinical infections and are not used for quarantine screening of animals.
- Conventional or Taqman polymerase chain reaction (“PCR”) tests are considerably more sensitive than morphologic methods and are useful for detecting the much smaller numbers of organisms present in subclinical infections.
- PCR is the test of choice for screening wild or captive populations for the presence of Bd infection and for quarantine screening of animals.
- Fungal culture, using techniques for conventional fungal organisms, is not useful for detecting Bd (Bd requires specialized techniques for culture).

Morphologic Methods to Detect Bd

The morphologic methods of diagnosis rely on identification of characteristic fungal bodies (thalli) of Bd by light microscopic examination of:

- shed skin.
- skin samples obtained by skin scraping.
- histologic sections.

For most species sampling of the ventral body surfaces is preferable because Bd thalli are present in higher numbers in these locations. The methods commonly used for morphologic diagnosis are:

- **Wet Mount.** Samples of shedding skin are obtained by gently scraping the ventral body surfaces or feet using the dull side of a scalpel blade, toothpick or sterile plastic spoon. See Figure 7.1.

The sample is:

- Spread out on a glass microscope slide with a small amount (1–2 drops) of water or normal saline and a coverslip applied.

- Using a light microscope with the condenser racked down the slide is examined for Bd thalli (see Figure 7.1). Good photomicrographs of unstained chytrid thalli are available at:
www.umaine.edu/chytrids/Batrachochytrium/Photographs/htm
- Stains such as cotton blue (Parker™ Ink) and 10% KOH (Mazzoni et al., 2003) and Congo Red (Briggs and Burgin, 2004) can be applied to help visualize the walls of Bd thalli.
- **Cytology.** Samples of skin are obtained as described for the wet mount technique. Skin samples are spread out on a microscope slide, air-dried, and stained with hematologic dyes such as Wright's or Wright-Giemsa (also Diff-Quik). See Figure 7.2.

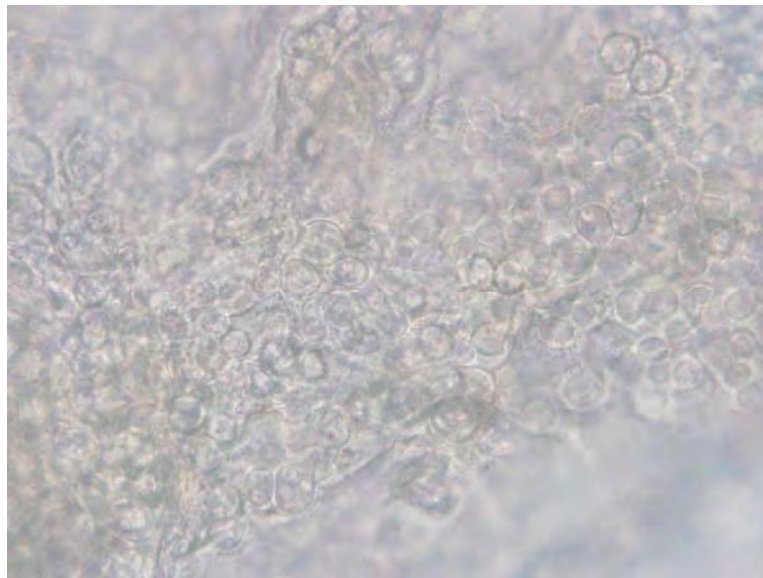


Figure 7.1: Wet mount of the skin from a White's Tree Frog (*Litoria caerulea*) examined by light microscopy. Numerous round structures representing the thalli of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) are present within skin cells.

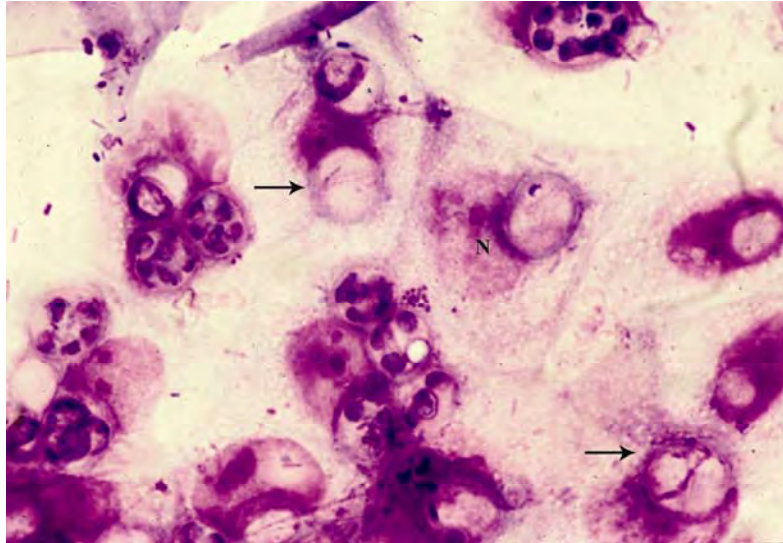


Figure 7.2: Cytology of the skin from a poison dart frog (*Dendrobates* sp.). Numerous round structures representing the thalli of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) are present within skin cells. Many thalli have internal round purple structures (zoospores), but other thalli are empty and appear as clear round spaces (arrows). The thallus at the bottom right hand corner has evidence of internal septation (colonial thallus). Reprinted from Pessier (2007).

- **Histology.** Diagnosis by histology is most applicable to skin samples collected at necropsy, however, toe clips and fragments of shed skin from living animals can also be processed in this fashion.
 - Samples for histology are preserved in 10% neutral buffered formalin (see Chapter 9 for the formulation of neutral buffered formalin) or 70–95% ethanol.
 - Necropsy samples should include multiple full-thickness sections of skin from the ventral body including the pelvic region (“drink” or pelvic patch), legs and feet.
 - For small animals (< 5–10 grams), the carcass is left intact, demineralized after formalin fixation and multiple whole body sections are prepared that include the skin.
 - Samples from anuran tadpoles should focus on the keratinized oral disc (“mouthparts”).
 - Routine staining with hematoxylin and eosin is usually sufficient to demonstrate thalli of Bd. The walls of thalli also stain with periodic acid-schiff (PAS) and Gomori methenamine silver (GMS).
 - Immunohistochemistry using polyclonal antibodies against Bd can be used to improve the sensitivity of histology, especially for subclinical infections (Van Ells et al., 2003). This method is not widely available and less widely used with increased availability of PCR diagnostics.
 - Histopathologic lesions in the skin associated with chytridiomycosis include:

- Hyperkeratosis.
- Epidermal hyperplasia.
- Variable degrees of inflammation, epidermal degeneration (often minimal).
- Secondary bacterial and fungal infections are common.

Morphologic features of Bd thalli. All of the morphologic techniques described depend on microscopic identification of characteristic thalli of Bd within the cytoplasm of skin cells (keratinocytes). Detailed references that describe morphologic features of Bd thalli are available (Pessier et al., 1999; Longcore et al., 1999; Berger et al., 2005). Features that may be most useful include (see Figure 7.3):

- Thalli usually measure 7–20 μm in histologic section. Four forms can be routinely identified:
 - Small uninucleate stage
 - Multinucleate form with stippled to microvacuolated cytoplasm
 - Mature zoosporangium that contains multiple discrete 2-3 μm basophilic spherical zoospores
 - Empty thalli (these have previously discharged zoospores). Empty thalli are sometimes the predominant forms observed by morphologic methods.
- The appearance of mature zoosporangia can be confused with life-stages of a protozoal organism. Practical features most helpful for definitive morphologic diagnosis are:
 - The presence of empty thalli that show evidence of internal septation. These are remnants of “colonial thalli” characteristic of Bd.
 - Observation of thalli with prominent discharge tubes that give the thallus a “flask-like” appearance.
 - In some cases (but not all) GMS staining of histologic sections can demonstrate the rhizoids, which are thin, root-like extensions from thalli.

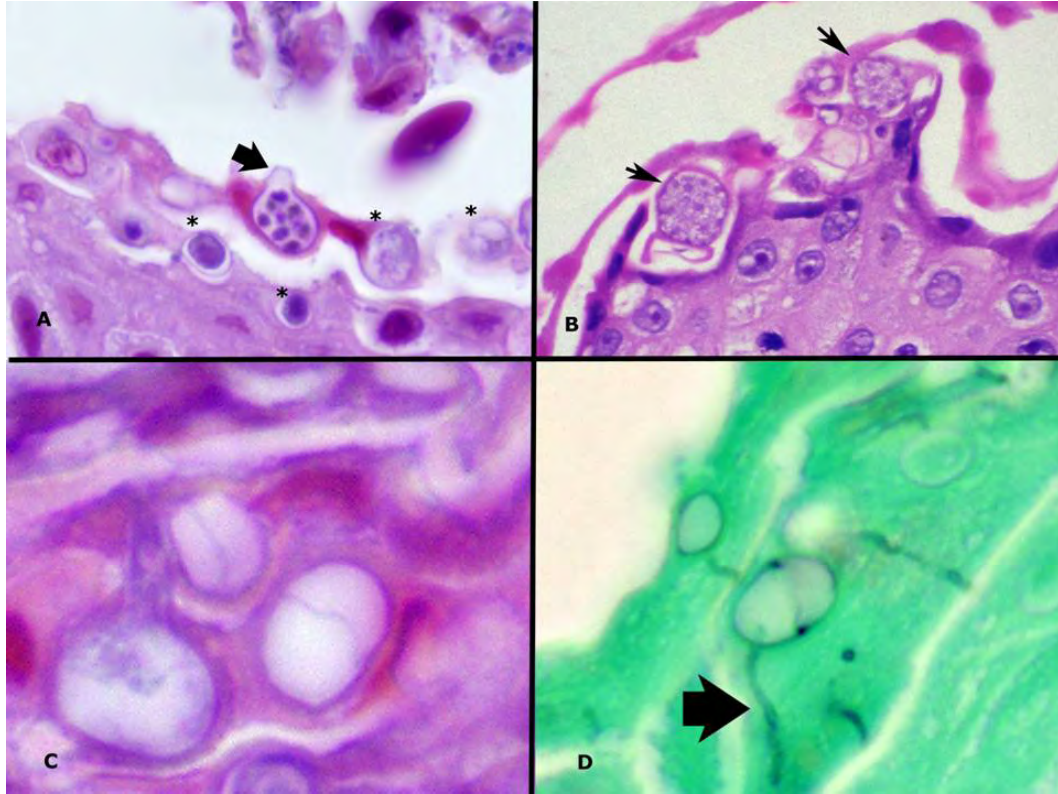


Figure 7.3: Detailed morphologic features of *Batrachochytrium dendrobatidis* thalli useful for histopathologic diagnosis: A) Mature zoosporangium with discrete basophilic zoospores and a discharge tube (arrow) and there also are several uninucleate developing thalli (asterix); B) Developing multinucleated thalli (arrows); C) Three “empty” thalli that have previously discharged their zoospores. The thalli in the center are “colonial” thalli as indicated by fine internal septation that appears to divide the thallus in half; D) A Gomori’s methenamine silver (GMS) stained section demonstrating the rhizoids (arrow), which are thin root-like projections from the thallus that are intermittently observed in silver stained histologic sections.

PCR-based Methods for Diagnosis of Bd

Polymerase chain reaction (PCR)-based tests are important diagnostic tools for the diagnosis of Bd infection. Both conventional (Annis et al., 2004; Goka et al., 2009) and real-time (Taqman) PCR techniques are in use (Boyle et al., 2004; Hyatt et al., 2007; Garland et al., 2009). PCR is the diagnostic method of choice for:

- Quarantine screening of new animals.
- Screening of animals prior to reintroduction or translocation.
- Surveys of captive and free-ranging populations for the presence and prevalence of Bd infection.
- Confirmation of positive results from clinical wet mount or cytologic examinations.

PCR methods are very sensitive and the Taqman method can experimentally detect as little as a single *Bd* zoospore (Boyle et al., 2004).

- Because of this exquisite sensitivity, a positive PCR result means only that an animal is infected with *Bd* and does not distinguish between a subclinical infection and a clinically significant infection (chytridiomycosis).
- Recognizing the distinction between subclinical infection and clinically significant infection is important when investigating amphibian mortality events. It means that positive *Bd* PCR results alone cannot prove that chytridiomycosis was the cause of an observed die-off.

Comparison of Different PCR Techniques. The real-time (Taqman) PCR technique (Hyatt et al., 2007) has advantages over the conventional PCR method (Annis et al., 2004). These advantages include:

- Greater test sensitivity.
- Reduced potential for cross-contamination during sample processing (resulting in false-positive tests).
- No need to confirm positive results by DNA sequencing of the PCR products.
- Use of internal controls that detect PCR inhibitors. Detection of PCR inhibitors is important because amphibian skin swab samples submitted for *Bd* PCR are frequently contaminated with inhibitor-rich dirt or plant material. Without internal controls for inhibitors, some samples that are actually positive for *Bd* DNA may be reported as negative (false-negatives).

Disadvantages of the real-time PCR technique are:

- Higher costs of necessary reagents.
- Limited availability of the more specialized equipment needed to perform this assay, and technique does not detect all strains of *Bd* due to the high specificity of the probe (Goka et al., 2009).

The choice of the PCR technique used will depend on a variety of factors including costs and availability of the different assays. A wide variety of different commercial and research laboratories are now offering PCR for *Bd*. Suggestions for evaluating and choosing a laboratory to perform *Bd* testing are provided in Section 7.2. The real-time PCR technique, if available, is preferred and likely will form the basis for international standards related to diagnostic testing for *Bd*.

Sample Collection for Bd PCR

The PCR procedure can be performed using a variety of different sampling methods including skin swabs, water bath, and tissue samples (toe clip or excision of tadpole mouthparts; Hyatt et al., 2007).

- The skin swab procedure is simple, minimally invasive and samples multiple areas of the skin that may be infected with Bd (increasing the likelihood that infected areas will be sampled). Skin swabs generally are the preferred sampling method for Bd PCR.
- Samples using the water bath procedure require immediate centrifugation or micropore filtration and are not practical in many settings.
- Toe clipping is an invasive procedure with associated ethical concerns and has the disadvantage of sampling only a small portion of potentially infected skin.

Materials Needed. The materials listed below are general guidelines needed to perform the skin swab procedure for Bd PCR using the Taqman method. There may be differences depending on the preferences of the laboratory processing the samples and the environmental conditions under which the swabs are obtained.

- Powder-free latex or nitrile disposable gloves.
- Sterile applicators (“swabs”); see “Swab Selection” below.
- 1.5 ml microcentrifuge tubes/cryovials.

Collection of samples for the conventional PCR method has called for storage of swabs in 70% ethanol. This is not recommended for the Taqman PCR procedure. For more information see the section on “Storage of Skin Swab Samples” below.

Swab Selection. There are a variety of commercially available applicator sticks or swabs that differ in the composition of the swab tip (cotton or rayon), composition of the swab handle (wood, plastic or metal) and size of the swab tip (fine tip or standard).

- The type of swab used can affect the performance of the PCR test and should be carefully considered before sampling animals.
- For the Taqman PCR procedure, a fine-tipped rayon swab with a plastic handle manufactured by the Medical Wire and Equipment Co. has shown the best performance (Hyatt et al., 2007 and M. Schrenzel, San Diego Zoo, unpublished). This swab is available as:
 - MW 100-100 (Australia).
 - Dryswab™ Fine Tip MW113 (United States: www.mwe-usa.com).

If this swab is unavailable, other rayon tipped swabs with plastic handles are possible substitutes.

- Cotton-tipped swabs or swabs with a wooden handle should be avoided (if possible) for the Taqman PCR procedure. They can be associated with reduced recovery of Bd DNA and possibly even false-negative tests (M. Schrenzel, San Diego Zoo, unpublished).
- Possible false-negative results from wood-handled swabs may be due to introduction of PCR inhibitors into the reaction (see section below: “Avoiding PCR inhibitors in samples”). Some laboratories that perform the conventional PCR procedure suggest cotton-tipped swabs and wooden handles. Laboratory studies to determine if PCR inhibitors could result in false-negative reactions with this assay are needed.

Swabbing Technique for Post-metamorphic Animals

(Photos by E. Kabay, Zoo Atlanta)

1. Using a fine-tipped permanent marker label a cryovial with the species name and individual animal ID number.



2. Put on a new pair of disposable gloves for each animal that is swabbed (See section on “Avoiding Cross-Contamination of Samples” below).



3. Open the package containing the sterile swab. Make sure to avoid contact of the swab with work surfaces, hands or enclosure substrate.



4. Using a gentle sweeping motion swab the ventral skin surfaces. Make sure to include the feet (front and hind), thighs and abdomen (especially the drink or pelvic patch). Each area should be swabbed 3–5 times. Because Bd infection tends to be concentrated on ventral surfaces it is not necessary to swab the dorsal skin.



5. Allow the swab to air-dry. Do not allow the swab tip to contact work surfaces, hands or substrate.



6. Break or cut the tip of the swab off into the previously labeled cryovial.



7. Store air-dried swabs for the Taqman-PCR analysis at or below 23 °C (room temperature). See section describing “Sample Storage.”
8. For shipment to the laboratory, swabs should be sent by overnight or 2-day courier service on icepacks to guard against temperature extremes. Samples that have been previously frozen should be sent on dry ice to prevent thawing.

A video presentation of this technique for Taqman-PCR may be viewed here:
<http://amphibiaweb.org/chytrid/index.html>

Another video presentation, using wooden-stemmed swabs suitable for conventional PCR, may be viewed here: http://www.amphibianark.org/frog_gallery.html

Sampling Techniques for Tadpoles

- In tadpoles, infection with Bd is limited to the keratinized oral disc or “mouthparts” and sampling for PCR is directed to this area.
- Samples for PCR include removal of the mouthparts after euthanasia of the tadpole (lethal sampling) or in living tadpoles by swabbing of the mouthparts using an applicator or wooden toothpicks (Retallick et al., 2006; Hyatt et al., 2007).
 - Use of the toothpicks, while still an effective method, was associated with decreased test sensitivity when compared to the lethal sampling method (Retallick et al., 2006).
 - The swab technique was comparable to lethal sampling method except in very low-level infections (Hyatt et al., 2007).
- For lethal sampling, euthanasia is carried out as described in Section 8.6. The mouthparts are removed using a new sterile scalpel blade. Mouthparts are air-dried onto filter paper or stored in 70% ethanol prior to processing.
- Supplies and general procedures for collecting swab samples are similar to that described for post-metamorphic animals above. Special considerations are:
 - Swab samples are obtained by inserting an applicator swab into the mouth and twirling the swab several times (Hyatt et al., 2007).
 - Toothpick samples are obtained by scraping the tooth rows and keratinized beak with a wooden toothpick (Retallick et al., 2006). Samples are to be preserved in 70% ethanol.

- There are concerns about potential toxicity of nitrile and latex gloves to tadpoles. Guidelines for minimizing this risk are available (Greer et al., 2009).

Avoiding Cross-contamination of Samples

The PCR assays are very sensitive tests and can detect very small amounts of Bd DNA. This is good for detecting animals that have very low-level infections with Bd, but increases the likelihood that samples from a non-Bd infected animal can become contaminated with Bd DNA from an infected animal, resulting in a false-positive test result. Therefore it is very important to take precautions to avoid sample cross-contamination which include:

- A new pair of disposable latex or nitrile gloves should be used for each animal handled for testing (Mendez et al., 2008).
- Avoid contact of swabs (especially swab tips) with surfaces or substrates other than the skin of the animal to be tested.
- If instruments are used to cut the tip of the swab into cryovials, a freshly disinfected instrument must be used for each sample.
 - To disinfect instruments for this purpose, dip in 70% ethanol followed by flaming under an alcohol lamp.
 - Avoid using bleach solutions for disinfection because this can degrade Bd DNA in swab samples (resulting in false-negative tests; Cashins et al., 2008).

Avoiding PCR Inhibitors in Samples

Foreign material such as dirt or plant matter can contain materials that inhibit the PCR reaction. This can result in a false-negative test result (animal is infected with Bd, but is not detected by the PCR test).

- Prior to skin swabbing efforts should be made to manually remove heavy skin contamination. Animals may be gently rinsed with clean water prior to sampling, but vigorous washing should be avoided because of the potential to also rinse off Bd infected skin cells or organisms.
- If rinsing with water is used for cleaning the water should not originate from the animal's enclosure or environment.
- Laboratories that perform PCR for Bd should always use exogenous internal positive controls to detect PCR inhibitors (Hyatt et al., 2007).

Storage of Skin Swab Samples

Storage of swabs after sample collection is an important consideration. The major concern for air-dried swabs is high temperature extremes:

- The DNA of Bd on air-dried skin swabs is remarkably stable and experimentally swabs have been stored for up to 18 months at room temperature (23°C) without a reduction in the sensitivity of the assay (Hyatt et al., 2007).

- In contrast, exposure of swabs to very high temperatures (> 38°C) for 7 days resulted in decreased recovery of Bd DNA that could result in false-negative results for animals with low-level Bd infections (Van Sluys et al., 2008).

Therefore, it is recommended that air-dried skin swab samples be stored at as low a temperature as possible (Skerratt et al., 2008).

- At 25°C (refrigerator) or lower.
- Samples should be frozen (–20°C or below) if sample analysis is not performed within six months of sample collection.

For shipment to the laboratory:

- Ideally ship swabs by overnight or 2-day courier service (e.g., Federal Express; UPS; DHL).
- Consider using cold packs to guard against temperature extremes (especially when there is the potential that packages will be exposed to high temperatures).
- Samples that have been previously frozen should be sent on dry ice to prevent freeze-thaw cycles.

Storage of swabs in 70% ethanol has been suggested as an alternative to low temperature storage, especially for swabs that will be analyzed by the conventional PCR method or field collected samples in which access to refrigeration is impossible (Annis et al., 2004; Brem et al., 2007). Details are available online at: www.amphibianark.org/pdf/Field%20sampling%20protocol%20for%20amphibian%20chytrid%20fungi%201.0.pdf. It would be valuable to have the ethanol preservation technique validated experimentally in the laboratory for preservation of samples exposed to high heat.

Pooling of Skin-swab Samples

Pooling or batch testing of skin swabs from multiple animals into a single PCR reaction has been used to reduce the costs associated with testing large numbers of animals for Bd. Batch testing is most useful for screening of populations where results from individual animals are not as important.

- For instances where results from individual animals are critical the pooling of swabs is not suggested. This is because test sensitivity may be reduced and the possibility that low-level Bd infections might be missed (false-negative test).
- Experimentally, up to 5 swabs have been combined without an overall loss of test sensitivity (Hyatt et al., 2007). However, in the same report a single swab from a field study with very low Bd levels was not detected when swabs were pooled and others have had similar observations (Skerratt et al., 2008).
- Experimental studies that examine the possibility of sampling multiple animals with the same swab should be conducted. This would only be useful for groups of

animals housed in the same enclosure and individual animal results are not important (animals housed together are assumed to be infected with Bd, regardless of individual animal PCR results).

Interpretation of Bd PCR Results

Results of Taqman PCR for Bd will usually be reported as positive, equivocal or negative.

- A positive result indicates that the test has detected the DNA of Bd and the animal is probably infected.
 - False-positive results are uncommon unless there has been DNA contamination either during sample collection (see “Avoiding Cross-Contamination of Samples” above) or during laboratory processing.
 - Laboratories performing PCR should be using procedures that reduce the possibility of contamination as well as negative controls that can detect contamination events (see Section 7.2).
 - Captive animals can be treated with antifungal drugs to attempt to clear infection (see Section 8.1). They should not be introduced into a zoo collection, a survival assurance colony or used in a reintroduction program until there is confidence that infection has been cleared.
- An equivocal result will be reported from laboratories performing the Taqman PCR test and occurs when 1 or 2 wells in an assay performed in triplicate are positive and other wells are negative for the DNA of Bd.
 - Equivocal results occur either from DNA contamination in the laboratory or from with low-level (subclinical) Bd infections.
 - The original sample may be re-tested, a recommendation may be made to re-sample the animal, or both (Hyatt et al., 2007).
- A negative result indicates that the test has not detected the DNA of Bd. Negative results can mean 1 of the following:
 - A true negative result because the animal is not infected with Bd
 - A false-negative result because the animal has a low-level infection with Bd (or has recently shed off a large amount of infected skin) and insufficient Bd DNA was present on the swab sample to be detected by the test.
 - A false-negative result from an animal infected with Bd because PCR inhibitors are present in the sample (see “Avoiding PCR Inhibitors” above). Most laboratories performing Taqman PCR are using internal controls designed to detect samples that contain PCR inhibitors.
 - Because the animal is infected with a strain of Bd that is not detected by the Taqman PCR (Goka et al., 2009).

The possibility of false-negative results, especially because of low-level infections, partially explains observations that naïve susceptible animals exposed to Bd will be detected as infections progress in intensity (Hyatt et al., 2007). It is possible that animals with chronic,

low levels of infection (disease carriers) may not be detected with this sampling regime. It may be necessary to stress animals, sample over a longer period or introduce naïve susceptible animals in order to detect Bd in carrier animals.

- In view of these observations, for situations where the results of PCR testing are critical (e.g., amphibian quarantine and reintroduction programs) multiple PCR swab samples over a 2-week period are suggested to increase confidence in any single negative PCR result.

An experimental study demonstrated that three swabs obtained over a 14-day period increases the likelihood that animals infected with Bd at a low level will be detected (Hyatt et al., 2007). This number of samples may not be possible from a logistic or financial standpoint for many programs that maintain captive amphibians. The decision to perform multiple tests will depend on the relative risk. Examples of higher risk situation include:

- animals obtained from collections with an uncertain health history or from dealerships that do not have Bd-free animal colonies.
- animals coming from collections or sources with recently identified cases of chytridiomycosis.
- animals coming into captivity from the wild.
- instances of very valuable animals where the risk (however small) of introducing or not detecting Bd is considered to be unacceptable.

7.4 RANAVIRUS INFECTION

Iridoviruses in the genus *Ranavirus* are recognized as a major cause of mass mortality events in wild amphibian populations (Green et al., 2002; Jancovich et al., 1997; Bollinger et al., 1999; Gray et al., 2009).

- Unlike chytridiomycosis, *Ranavirus* infections have not been conclusively associated with long-term population declines.
- In captive animals or zoo amphibian collections, *Ranavirus*-associated mortality events have only very recently been documented (Donnelly et al., 2003; Majji et al., 2006; Pasmans et al., 2008; Miller et al., 2008) and very little is known about the prevalence and significance of infection in captive populations.
 - This may be because the clinical and pathologic findings can overlap with other amphibian infectious diseases and because specific diagnostic tests have not always been readily available to veterinarians.
- *Ranavirus* infections can be subclinical (carrier animals with no outward signs of disease) and therefore infections could occur in captive situations without detection.
- Prospective surveys of captive amphibian collections for evidence of *Ranavirus* infection are necessary to better inform captive management and amphibian reintroduction programs.

Major concerns about ranaviruses in relation to amphibian survival assurance colonies include:

- The potential to cause significant mortality in vulnerable captive populations.
- The potential that captive-reared amphibians from cosmopolitan zoo collections could serve as a vector for movement of ranaviruses into vulnerable wild amphibian populations.
 - Anthropogenic movement of ranaviruses has been documented for the fish bait trade in the United States as well as bullfrogs imported to the United States (Picco and Collins, 2008; Schloegel et al., 2009).
- Some ranaviruses have a broad host range that can include not only different amphibian species, but also different classes of animals especially fish, amphibians and reptiles expanding the potential ecological importance of ranaviruses introduced to new locations (Johnson et al., 2007; Schock et al., 2008).
- There can be important intraspecific differences among pathogen strains and host populations. Even closely related strains of ranaviruses (e.g., strains of *Ambystoma tigrinum* virus) can cause very different levels of mortality and disease. Further, different populations of amphibians can respond very differently to a single strain of a pathogen. That is to say, not all strains behave the same way, and not all host populations behave the same way (Schock et al., 2010).

The ranaviruses are a large group of related viruses that may each have unique biological behavior (e.g., host range; virulence to different amphibian species). The ranaviruses of concern to amphibian conservation will most often fall into the Frog Virus 3 (FV3)-like viruses, *Ambystoma tigrinum* (ATV)-like viruses or the Bohle iridovirus (BIV).

- The FV3-like viruses are most often isolated from anuran amphibians (frogs), but have also been found in turtles and some salamanders (Johnson et al., 2007; Duffus et al., 2008).
- The ATV-like viruses are found naturally in salamanders with anuran amphibians appearing to be resistant to infection. However, recent experimental infections of ranid frogs have raised the possibility of a broader host range for the ATV-like viruses (Schock et al., 2008).
- The Bohle iridovirus (BIV) from Australia is distinct from both the FV3 and ATV viruses and is important because it has been shown to cause disease in both anuran amphibians and fish.

Outbreaks of ranaviral disease in anurans with FV3-like viruses often occur in tadpoles or recently metamorphosed froglets, but disease can also be observed in adult animals. Disease associated with ATV-like viruses in salamanders can be observed in both larval and adult life stages.

- Clinical signs of ranaviral disease can include cutaneous (skin) hemorrhage, skin ulceration, visceral hemorrhage and subcutaneous edema. Infections can outwardly resemble the “red leg syndrome” historically attributed to systemic bacterial infections in amphibians.
- Epidermal hyperplasia resulting in skin “polyps” may be observed in salamanders infected with ATV-like viruses.
- In anurans, chronic skin ulceration without evidence of systemic ranaviral disease has been observed (Cunningham et al., 2008).

Subclinical infections (no outward signs of disease) do occur. But it is unclear if these are transient or can be persistent for long periods of time. Potential duration of subclinical infections may depend on host species, the type of *Ranavirus* and other factors such as immune status.

- Experimentally infected Clawed Frogs (*Xenopus laevis*) cleared infection with Frog Virus-3 in as little as 20 days, however, a group of tiger salamanders subclinically infected with *Ambystoma tigrinum* virus had evidence of infection for at least 6 months (Brunner et al., 2004; Robert et al., 2007).

Selection of a Diagnostic Test for Ranaviruses

Diagnosis of *Ranavirus* infection is easiest in animals that are sick and have systemic ranaviral disease. Unlike PCR testing for the amphibian chytrid fungus, the available diagnostic tests are not validated for detecting infections in outwardly healthy animals (subclinically infected animals). This means that there are no reliable tests for detecting subclinical infections in living animals for purposes such as:

- Surveys of wild or captive populations for occurrence or prevalence of *Ranavirus* infection.
- Screening new animals in quarantine prior to entry into a captive collection.
- Screening prior to use in a reintroduction or translocation programs.

A variety of diagnostic methods including histopathology, immunohistochemistry, virus isolation in cell culture, molecular methods (polymerase chain reaction) and serologic tests are described in the research literature and are summarized below. For disease diagnosis, infectious disease surveillance and risk assessment for captive amphibian conservation programs, polymerase chain reaction (PCR) based testing is most commonly used to definitely diagnosis *Ranavirus* infections.

Morphologic Methods of Diagnosis for Ranaviruses

Necropsy and histopathology can be used to develop a preliminary diagnosis of *Ranavirus* infection if suspicious microscopic lesions combined with or without characteristic inclusion bodies are observed. Some common lesions associated with *Ranavirus* infection include:

- Necrosis with or without hemorrhage in sites such as the liver, kidney, gastrointestinal tract, hematopoietic tissue (including sites of hematopoiesis in the liver, kidney and spleen) or skin (Gray et al., 2009).
- Epithelial proliferation (skin) in ATV infection (Bollinger et al., 1999)
- Skin ulceration may sometimes be the only finding (Cunningham et al., 2008)
- Intracytoplasmic basophilic inclusion bodies (see Figure 7.4 below). These are sometimes difficult or impossible to detect. In some lesions they can be confused with nuclear or other cell debris.
- Subtle or non-specific lesions such as diffuse hematopoietic tissue necrosis that may not be recognized unless the pathologist has extensive amphibian histology experience.

Because findings can be non-specific and overlap with other infectious diseases of amphibians, confirmation of a suspected *Ranavirus* diagnosis by another diagnostic method such as immunohistochemistry or PCR usually is needed.

- Immunohistochemistry has been successful using rabbit antiserum to the Epizootic Haematopoietic Necrosis Virus (EHNV) of fish (Cunningham et al., 2008) or a monoclonal antibody to the major capsid protein of FV3 (Robert et al., 2005). Availability of these reagents may be limited to research laboratories.
- A technique for amplifying *Ranavirus* DNA from paraffin-embedded tissue has been described (Kattenbelt et al., 2000).
- Transmission electron microscopy can be used to demonstrate iridovirus virions in affected tissues.

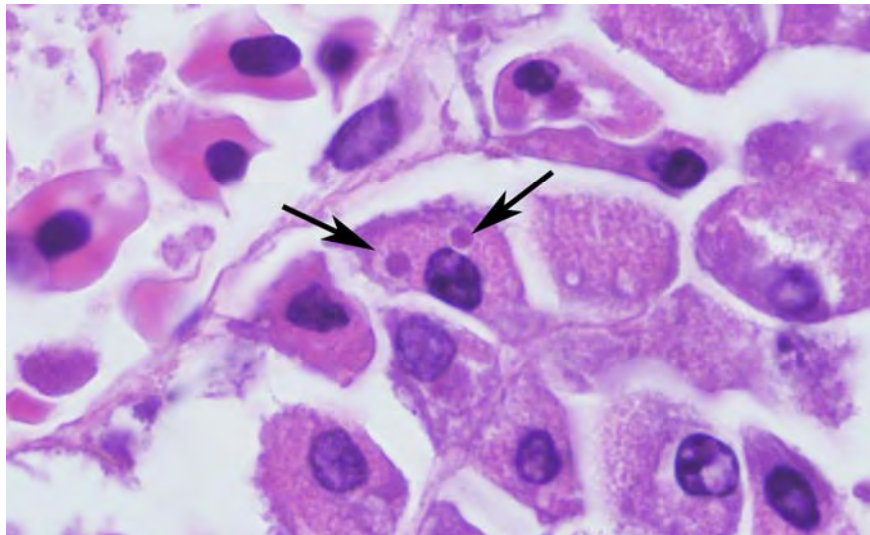


Figure 7.4: Histologic section of the liver showing characteristic intracytoplasmic inclusion bodies of ranavirus infection (arrows) within hepatocytes (liver cells).

Isolation of Ranaviruses in Cell Culture

Ranaviruses can be isolated in cell culture and fish cell lines are commonly used (Hengstberger et al., 1993; Zupanovic et al., 1998; Bollinger et al., 1999; Docherty et al., 2003).

- Virus isolation is most likely to be used in a research laboratory setting and may not be widely available for clinical diagnosis of *Ranavirus* infection.
- Virus isolation is required to perform restriction fragment length polymorphism (RFLP) profiles necessary to identify a specific *Ranavirus*. This may be useful in some amphibian conservation programs where it becomes necessary to know if the virus circulating in a wild population is the same as a virus present in a captive population (see below).
- Suitable samples for virus isolation are fresh or frozen tissues such as liver and kidney obtained at necropsy from affected animals.

Serology for Ranaviruses

An enzyme linked immunosorbent assay (ELISA) technique has been described for the detection of anti-*Ranavirus* antibodies in sera from marine toads (Zupanovic et al., 1998).

- This technique detects only prior exposure to a *Ranavirus* and not active infection.
- ELISA testing might be useful for population-level health surveys; however, the test is not available outside of research laboratories.
- Other disadvantages to more widespread application of this testing method are the inability to obtain large blood sample volumes from many captive species and the need to validate the test for species other than the Marine toad (*Rhinella marina*).

PCR-based Methods for Diagnosis of Ranavirus Infection

Polymerase chain reaction (PCR)-based tests for detecting the DNA of ranaviruses are becoming more widely available outside of research laboratory settings. Both conventional and real-time (Taqman) PCR techniques based on the major capsid protein (MCP) gene have been described (Mao et al., 1997; Pallister et al., 2007; Schock et al., 2008).

- The PCR techniques are best validated for use on tissue samples. They can be applied to other sample types (e.g., swabs from living animals), but results need to be interpreted with caution.

Comparison of Different PCR Techniques. Conventional PCR using the MCP 4/5 primer set described by Mao et al., 1997 is commonly used to detect ranaviruses.

Advantages of the conventional PCR test are:

- Ability to perform DNA sequencing of the PCR product and obtain some additional information on the type of *Ranavirus* present (e.g., FV3-like or ATV-like virus).
- Low cost for reagents and equipment compared to real-time PCR methods.

Disadvantages of the conventional PCR test include:

- A need to perform DNA sequencing or Southern blot analysis to confirm positive results.
- Conventional PCR may be less sensitive than real-time PCR techniques.

The real-time PCR technique has advantages for diagnostic situations such as:

- No need to confirm positive results by DNA sequencing or Southern blot analysis. This speeds the availability of diagnostic information.
- Increased sensitivity of real-time PCR allows for detection of smaller amounts of *Ranavirus* DNA.

Disadvantages of real-time PCR are:

- Higher costs for reagents and equipment.
- If there is a need to further characterize the virus (e.g., determine FV3-like vs. ATV-like virus), additional PCR reactions with more specific real-time PCR primer sets or conventional PCR with DNA sequencing will be required.

Sample Collection for *Ranavirus* PCR

The choice of sample type for *Ranavirus* PCR will depend on if samples are obtained at the time of necropsy from a dead animal or if sampling is attempted on a living animal.

Necropsy Samples:

For testing of sick animals that die naturally or are euthanized because of suspected *Ranavirus* disease. Lethal *Ranavirus* infections affect multiple organ systems and therefore sampling of one or more of the following tissues at necropsy should be diagnostic:

- Liver.
- Kidney.
- Skin (if ulcerative or proliferative skin lesions are observed; concurrent PCR of liver or kidney is suggested for most cases).

For using necropsy tissues to detect animals are subclinically infected with a *Ranavirus* (the animal did not die of *Ranavirus* infection, but was carrying an infection). This type of testing may be useful for testing populations for the presence or absence of *Ranavirus* infections.

- Kidney and tissues that contain high numbers of resident macrophage-type cells such as liver and spleen may be useful based on findings in Clawed Frogs (*Xenopus laevis*) subclinically infected with FV3 (Robert et al., 2005, 2007).
- Pulverization and PCR analysis of entire carcasses is another consideration for population surveys (Greer and Collins, 2007).

Necropsy tissue samples are collected into Whirl-Pak® style bags (Nasco, USA, www.enasco.com) or cryovials (Nunc CryoTubes™ or Vangard Cryos™ (Sumitomo Bakelite Co., Ltd., Japan; www.sumibe.co.jp/english/). A separate set of instruments is used for each tissue sampled to avoid cross-contamination. Tubes are labeled with the species name, tissue type, individual animal ID number and date.

Clinical or Non-lethal Samples (Samples from Living Animals):

Because lethal *Ranavirus* infections usually affect multiple organ systems, different types of clinical (nonlethal) samples have the potential to be successful in detecting infection.

- Clinical samples are most likely to be successful in detecting infection in animals evidently sick with ranaviral disease. These animals often have large amounts of virus detectable in multiple tissues.
- Unlike PCR testing for the amphibian chytrid fungus, the available diagnostic tests are not validated for detecting infections in outwardly healthy animals (subclinically infected animals). This means that there are no reliable tests for detecting subclinical infections in living animals for purposes such as:
 - surveys of wild or captive populations for occurrence or prevalence of *Ranavirus* infection.
 - screening new animals in quarantine prior to entry into a captive collection.
 - screening prior to use in a reintroduction or translocation programs.

Subclinically infected animals may have smaller amounts of virus present and virus may only be present in some tissues (e.g., kidney). These infections may therefore be harder to detect in the kinds of samples that are easily collected from a living animal. If outwardly healthy animals are tested, a positive test result that indicates the detection of *Ranavirus* DNA is more definitive than a negative test result. Negative test results from outwardly healthy animals should not be used to determine or declare that an animal is free of a *Ranavirus* infection.

The types of samples that can be considered for living animals include:

- Swabs of the skin (only if skin lesions are present such as ulcers), oral cavity/pharynx, or cloaca.
 - Plastic handled, rayon tipped swabs are preferable for collection of PCR samples.

- The swab is gently applied or swirled in the location to be sampled and after sample collection the tip of the swab is broken or cut off into a labeled cryovial or sterile bag.
- Oral and cloacal swabs were able to variably detect *Ranavirus* infection in experimentally infected Red-eared Slider Turtles (Johnson et al., 2007), but this has not been validated in amphibians.
- Tissue biopsy using toe clips (anurans) or fin clips (tail tips of salamanders) has been described as a non-lethal sampling method (St-Amour and Lesbarreres, 2007; Greer and Collins, 2007).
 - This is most likely to be diagnostic for animals that are viremic (large amounts of virus circulating in the blood).
 - Techniques are less sensitive than PCR of liver or whole body homogenates
 - May not detect animals in the early stages of infection or animals with subclinical infection.
 - Tissue biopsy may be too invasive for routine clinical use.
- Whole blood or blood buffy coat samples might be useful for detecting viremic animals.
 - This technique would not be expected to be diagnostic for animals in the early stages of infection or animals with subclinical infection.
 - The ends of centrifuged microhematocrit tubes containing the buffy coat are broken off into a cryovial or tubes are stored in a sterile plastic bag.
 - Sodium citrate or EDTA are preferable if anticoagulants are used. If blood is collected into vials with anticoagulants, be sure to notify the lab that is running the tests. The presence of these anti-coagulants can interfere with PCR reactions and create false negative results. However, these anticoagulants can easily be handled in PCR reactions as long as the laboratory is aware of the presence in the samples and can make the necessary adjustments to PCR reaction conditions.

Storage of Samples:

All samples for *Ranavirus* PCR should be stored at as low a temperature as possible after collection.

- Freezing in a conventional freezer (-20°C) is adequate for short-term storage of 1–2 weeks. Be careful to avoid (or disable) automatic defrosting cycles, available on many models of freezers. Defrosting will compromise the quality of the samples.
- For longer-term storage it is best to freeze at temperatures of -70°C or below with an ultra-cold freezer or liquid nitrogen.
- Frozen samples should be shipped to the laboratory on dry ice to prevent thawing.

Interpretation of *Ranavirus* PCR Results

The results of both conventional and real-time PCR techniques for *Ranavirus* are usually reported as “positive” or “negative.”

- A positive PCR result indicates that the test has detected *Ranavirus* DNA in the sample. A positive PCR result alone indicates only the presence of a *Ranavirus*, but does not distinguish animals that had lethal infections from subclinical carriers of infection. Correlation of PCR results with clinical and necropsy (histopathologic) findings typical of *Ranavirus* infection (see above) are necessary to determine if *Ranavirus* was the likely cause of death or of a mortality event.
- False-positive PCR tests are circumstances where there is a positive test result, but the animal is not infected with a *Ranavirus*. Most commonly, this will occur as the result of:
 - Contamination of samples with *Ranavirus* DNA during sample collection or during laboratory processing. All laboratories that perform PCR should have procedures in place to reduce the possibility of contamination and should use negative sample controls to detect contamination events.
 - Not performing confirmatory DNA sequencing or Southern blot analysis on positive samples as determined by the conventional PCR technique. This is because non-*Ranavirus* DNA will sometimes be amplified by the PCR reaction and appear as a band on an agarose gel that is approximately the same size as the band expected for *Ranavirus*. The real-time PCR technique has an internal probe that confirms amplification of the correct DNA sequence and therefore DNA sequencing is not required to confirm positive results.
- A negative PCR result indicates that the test has not detected *Ranavirus* DNA in the sample submitted to the laboratory. This is not always indicative that the animal is uninfected by *Ranavirus*; this latter declaration can be complicated by a false-negative result.
- A false-negative result (animal is infected with a *Ranavirus*, but the PCR test is negative) because:
 - Virus DNA was not present in the sample submitted to the laboratory. This is most likely to occur in samples such as swabs (pharyngeal or cloacal), tissue biopsies, or blood (buffy coat) samples taken from living animals. These animals may not be viremic or shedding virus into the locations sampled. Although most *Ranavirus* infections can be detected in multiple tissue types, sometimes they are limited to a single location such as the skin or the kidney and the sample submitted for testing is very important to avoid false-negative results.
 - Virus DNA is present in amounts below that detectable by the PCR method used. This situation is most likely to occur with subclinical infections. The real-time PCR methods are considered to be more sensitive and more likely to detect infections with small amounts of viral DNA.
 - PCR inhibitors are present in the sample. This is most likely to occur with cloacal swabs that may contain significant amounts of fecal material.

Specific Identification of Ranaviruses

In most situations for captive amphibian populations it will be sufficient to confirm a diagnosis of *Ranavirus* infection by PCR methods that are available in variety of research and commercial laboratories.

- These methods cannot determine the precise species of *Ranavirus* present in infected animals. Conventional PCR and sequencing will usually determine the broad group (e.g., Frog Virus 3-like or ATV-like) of *Ranavirus* present in an animal or group of animals.

Because a large number of different *Ranavirus* species can be placed in these broad groups (Schock et al., 2008), more precise characterization of the virus present is sometimes needed to make animal or population management decisions. For example, ranaviruses might be identified in both a wild population of frogs and in captive frogs of the same species that are housed in an amphibian survival assurance colony that is breeding animals for release back into the wild.

- If only conventional PCR and DNA sequencing are performed, it is likely that the virus in the wild population would receive a similar characterization as the virus present in the captive population (e.g., “Frog Virus 3-like”).
- This determination could lead to an erroneous conclusion that the virus in the captive population is the same as the virus in the wild population and that it is safe to introduce the infected captive reared frogs to the infected wild population. However, different species of *Ranavirus* that vary in virulence (ability to cause severe disease in different species) will have similar or identical profiles (Majji et al., 2006; Schock et al., 2010).
- More advanced measures to identify the species of viruses affecting both the captive and wild populations are required to better determine the likelihood that the viruses are the same species and to perform a good risk assessment for the reintroduction program.

Attempts to specifically identify a *Ranavirus* will require isolation of the virus into cell cultures (see above) followed by application of techniques, which include restriction fragment length polymorphism analysis (Hyatt et al., 2000; Majji et al., 2006; Schock et al., 2008).

- These techniques are most likely to be performed by research laboratories and may not be widely available.
- These types of molecular characterization require tissues that are frozen or fresh so that viable virions are still present. Samples preserved in ethanol cannot be used for these types of characterization.

7.5 PERKINSUS-LIKE PROTOZOAL DISEASE (ALVEOLATE PATHOGEN) OF RANID FROGS

Infection by an emerging protistan pathogen, referred to by some as the *Perkinsus*-like agent has resulted in mass mortality events in tadpoles of wild ranid frogs in the United States (Green et al., 2003; Davis et al., 2007). The lifecycle and modes of transmission have not been definitively determined.

- Affected species have included the Wood Frog, *Lithobates sylvaticus*; Southern Leopard Frog, *L. sphenoccephalus*; American Bullfrog, *L. catesbeianus*; Mink Frog, *L. septentrionalis*; green frog, *L. clamitans*; Dusky Gopher Frog, *L. sevosus*; and Gopher Frog, *L. capito*.
- Infections with the organism are not conclusively determined to be the cause of long-term population declines.
- There are concerns that mass mortality events associated with the organism may threaten the persistence of some species by reducing or eliminating natural recruitment in some ponds. There is particular concern for one of the most endangered anurans in North America, the Dusky Gopher Frog (*Lithobates sevosus*), with about 100 adults remaining in the wild.

Significant disease or mortality events have only been observed in ranid tadpoles.

- Subclinical infection (no evidence of disease or death) has been documented in some hylids such as the Spring Peeper (*Pseudacris crucifer*) and Southern Cricket Frog (*Acris gryllus*).
- Infections in postmetamorphic animals have been subclinical and limited to the lumen of the intestine with the exception of a small group of metamorphs of the Southern Leopard Frog *Lithobates sphenoccephalus* (Davis et al., 2007) that had infection of the liver similar to that observed in tadpoles.
- Except for wild animals brought into captive survival assurance colonies or research laboratories, infection with the *Perkinsus*-like agent has not been documented in captive animals or zoo amphibian collections.

The major concerns about the *Perkinsus*-like agent in relation to amphibian survival assurance colonies are:

- Uncertainty about the potential host range for this organism and the implications of introduction into zoo collections or survival assurance colonies.
- The unknown potential for introduction of this organism into naïve wild amphibian populations by movements of captive frogs in reintroduction programs.
- The potential for significant mortality in captive populations of susceptible species.

The life history of the *Perkinsus*-like agent is unknown and no experimental transmission studies or experimental infections have been achieved to date. Reported

clinical signs of infection in tadpoles are lethargy, bloating and occasionally cutaneous hemorrhage. Gross necropsy findings include markedly enlarged internal organs such as the liver, spleen and kidney that may be discolored white.

Diagnosis of Perkinsus-like Protozoal Disease

Diagnosis in evidently sick tadpoles is by morphologic identification of characteristic spores by light microscopy using smears of fresh tissues or by histopathology (see below).

- There is no reliable diagnostic test that can be used to detect the presence of infections in captive amphibian populations other than necropsy surveillance on sick or dead animals.

Diagnostic methods include:

Tissue smears:

Performed by taking a small piece of fresh tissue (2 x 2 mm) obtained at necropsy and placing it on a clean microscope slide. A second slide is then used to smear the tissue across the length of the first slide ensuring that the tissue is completely flattened (Davis et al., 2007). The slide is then examined by light microscopy for characteristic sub-spherical spores measuring approximately 6 x 5.5 microns (see Figure 7.5 below).

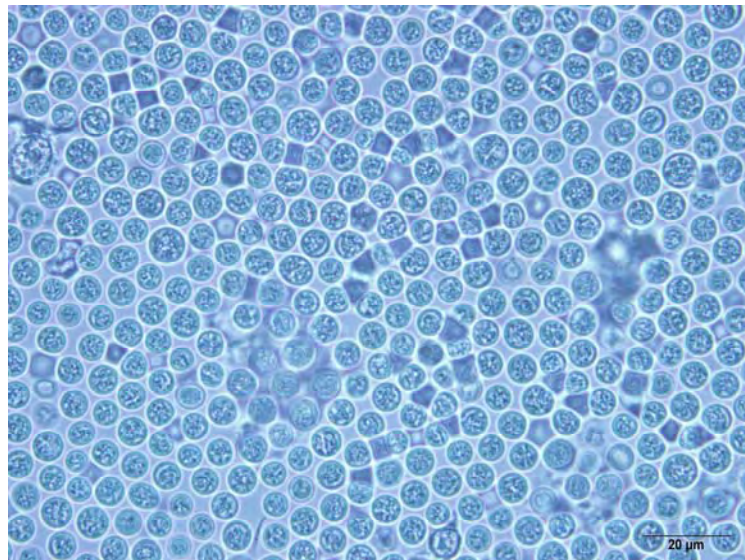


Figure 7.5: Photomicrograph of a tissue wet mount showing the characteristic *Perkinsus*-like protozoal spores.

Cloacal Wash or Examination of Intestinal Contents:

These materials are examined for spores as described for tissue smears. May be used in living animals, but probably should not be considered a definitive test for declaring animals free of infection.

Histopathology:

Necropsy is performed on tadpoles using the carcass fixation technique (see Chapter 9). Briefly, the tadpole is opened by making an incision into the coelomic cavity and the entire carcass is then immersed in a fixative solution (usually 10% neutral buffered formalin or ethanol). Tissues are processed routinely for histologic examination. On examination of the histologic sections, characteristic basophilic spherical spores are present in large numbers infiltrating tissues such as the liver, kidney, spleen, skeletal muscle, skin and mesentery.

Molecular Diagnostic Techniques:

A specific molecular diagnostic test is not widely available. A conventional nested PCR technique using primers that amplify the 18S rRNA gene of a wide range of protozoan eukaryotic organisms followed by DNA sequencing has been used (Davis et al., 2007).

7.6 DISEASE REPORTING AND PARTICIPATION IN DISEASE DATABASES**OIE Disease Reporting**

The World Association for Animal Health (OIE) is an international organization responsible for improving animal health worldwide and is recognized as a reference organization by the World Trade Organization (WTO). As of April 2009 there were 174 member countries and territories. A major objective of the OIE is “Sanitary Safety” by safeguarding world trade by publishing health standards for international trade in animals and animal products. These standards are recognized by the WTO as reference international sanitary rules.

Country signatories to OIE are required to report on their status for notifiable diseases to the OIE every six months. Currently two amphibian diseases are notifiable, chytridiomycosis and ranaviral diseases (Schloegel et al., 2010).

- The chief veterinary officer within a country has OIE reporting responsibility. Testing results for these diseases including negative results should be forwarded to the person within country who reports to the OIE (OIE, 2008). In the United States the chief veterinary officer would be the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA/APHIS).
- However, at this time the reporting responsibility for zoos or aquariums or amphibian survival assurance colonies in regards to the occurrence of chytridiomycosis or *Ranavirus* infection is unclear (at least for the United States). Guidelines for reporting or enforcement of OIE recommendations for disease screening are not in place.
- OIE listing of chytridiomycosis and ranaviral diseases will add requirements for infectious disease testing to international and possibly interstate shipments of amphibians. Limitations for the short term may include: lack of certified laboratories; costs for disease testing and creation of specific pathogen free

amphibian populations; lack of a good validated test for ranaviruses in living animals.

- OIE listing of chytridiomycosis and ranaviral disease may also require certification of assays and international reference standards used for testing for these diseases as they relate to international animal shipments. See the following links:
- www.oie.int/vcda/eng/en_background_VCDA.htm?e1d9
- www.oie.int/vcda/eng/en_fichier_SOP.pdf

Details on OIE requirements for international movement of amphibians under the 2009 Aquatic Animal Health Code for both chytridiomycosis and ranaviral disease can be obtained online at:

Chytridiomycosis: www.oie.int/eng/normes/fcode/en_chapitre_1.8.1.htm

Ranavirus: www.oie.int/eng/normes/fcode/en_chapitre_1.8.2.htm

Disease Databases

There are ongoing efforts worldwide to map the occurrence of significant infectious diseases of amphibians, especially infection with the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd). It is hoped that these efforts will improve the understanding of the distribution, movement, and genetics of Bd. As institutions, laboratories, and field researchers identify Bd infected amphibians it is hoped that they will report this information to the databases in order to gather as much information as possible. Concerns that may need to be addressed by available databases include standardization and validation of data generated by a variety of different sources, institutional privacy concerns and ownership of data.

The most comprehensive, updated database for chytridiomycosis is online: <http://www.spatialepidemiology.net/bd/>

7.7 TESTING AND SURVEILLANCE FOR FECAL PARASITES

Laboratory examination of feces to screen for presence of internal parasites is a common method of disease surveillance for the captive management of amphibians. Rudimentary fecal examination techniques require minimal equipment and can be used even in remote locations that have amphibian survival assurance colonies.

- Fecal examination detects protozoal parasites or various helminthes (cestode, trematode or nematode worms). The parasites detected are not limited to the intestinal tract. For example, the adult worms of the nematode *Rhabdias* are located in the lung of a variety of amphibians.
- Fecal examination detects both parasites that are likely to cause disease problems in captive amphibians and commensal organisms that are not associated with disease.
- Wild amphibians frequently (and normally) carry a variety of internal parasites, most the known parasites and parasitic diseases are not significant population limiting factors in wild populations. However, some of these parasites become important

disease problems in captive populations because of poor hygiene, inadequate husbandry and stress.

- Parasite surveillance is necessary for maintaining healthy captive populations. The rhabditiform nematodes, *Rhabdias* (amphibian lungworm) and *Strongyloides* (an intestinal worm) have become significant problems in many amphibian survival assurance colonies (Lee et al., 2006; Pessier, 2008; Gagliardo et al., 2008). Laboratory detection is an important part of a control program for these parasites.

Considerations when deciding on approaches to parasite monitoring and control in captive populations are:

- Elimination and control of all internal parasites in captive situations may not be possible or desired. For example:
 - The rhabditiform nematodes (*Rhabdias* and *Strongyloides*) are difficult to completely eliminate with anthelmintic treatment. Therefore, it can be better to emphasize reduction of parasite levels through treatment and maintenance of good enclosure hygiene, rather than using lengthy or repeated treatments to try to completely clear infection.
 - For animals in survival assurance colonies that will be returned to the wild, it may be desirable to maintain low levels of the parasites endemic in the wild populations of that species. There is a theoretical concern about loss of acquired natural resistance to these organisms needed for success after release (Lyles and Dobson, 1993; Cunningham 1996).
- It is important to differentiate between pathogenic or potentially pathogenic parasites and the commensal organisms that are common in amphibian gastrointestinal tracts (see below).

Techniques for Fecal Parasite Examination

The techniques used for fecal parasites examination are fecal wet mounts, fecal flotations, fecal sedimentation and the Baermann technique. All of these are described in detail in standard parasitology texts and are familiar to veterinarians (Foreyt, 2001).

- The most rudimentary techniques (fecal wet mount and passive flotation) for detecting fecal parasites require only a basic compound (light) microscope and simple reagents.
 - These techniques are helpful for amphibian colony health management in remote locations and are adequate for detecting rhabditiform nematodes which are the most common significant parasite problems in survival assurance colonies.
- There is a lack of baseline knowledge regarding the appearance of the eggs of amphibian parasites. This sometimes makes definitive identification difficult or impossible.

Fecal Wet Mount:

- This technique is also called the “fecal direct smear.”
- This technique is most useful for identification of motile parasites such as some species of protozoa and nematode larvae.
- Very fresh feces is required. If feces cannot be processed for laboratory examination immediately, they should be refrigerated (4 °C).
- Frequently used for detection of parasites in amphibians because only a very small sample of feces is required for analysis.
- Disadvantages of the wet mount are: 1) poor sensitivity for detecting low numbers of parasites eggs or oocysts in the sample; and 2) eggs or oocysts can be obscured by presence of fecal debris in the sample.
- A qualitative fecal scoring system used in the treatment of rhabditiform nematode infections is based on fecal wet mount examination (see Section 8.4).

To perform the fecal wet mount:

- 1) Apply a small amount (drop) of saline solution (0.9% sodium chloride) to a clean glass microscope slide.
- 2) Add a small amount of feces (size of a head of a match) to the drop of saline.
- 3) Mix feces and saline in a circular motion.
- 4) Place a coverslip on the slide and examine on 10x and 40x microscope objectives for parasites, parasite ova and oocysts.

Fecal Flotation:

- Many helminth parasite eggs can be concentrated by flotation. Concentration increases the sensitivity of the examination.
- This technique relies on use of a supersaturated solution that lifts eggs to the surface of the column of liquid due to their lower specific gravity. Eggs are collected at the surface and are less obscured by fecal material (compared to the fecal wet mount).
- Flotation techniques are passive (feces mixed with flotation solution) or centrifugal. Centrifugal techniques are more sensitive, but require a centrifuge and may not be practical for remote survival assurance colonies. More information on this technique may be found here:
<http://www.drmihaeldryden.com/parasitology/5/clinical-parasitology>

To perform the passive fecal flotation method:

- 1) Prepare a flotation solution. A solution with a specific gravity of 1.2–1.3 is best. If solutions will be prepared on-site a hydrometer is useful to be able to verify and adjust the specific gravity of flotation solutions. Common flotation solutions are:
 - Magnesium sulfate (Epsom salts) (400g dissolved in 1000ml water).
 - Sugar (454g granulated sugar + 355ml hot water to dissolve)

- Sodium nitrate (568g anhydrous NaNO_3 + 1000ml warm water to dissolve).
 - Zinc sulfate (33g anhydrous ZnSO_4 in distilled water; bring to 100ml).
- 2) Place 1–2g feces into a narrow flat bottomed tube or a commercially available fecal flotation vial (Fecalizer®, Vetoquinol USA; Ovassay, Sybiotics Corporation, USA).
 - For assessing fecal parasite problems in amphibian colonies, it is acceptable to collect and combine feces from several animals in the same enclosure in order to obtain a large enough sample for fecal parasite analysis.
 - 3) Add approximately 15 ml of flotation solution and mix well. Commercially available flotation vials have a built in strainer that removes fecal debris. If non-commercial vials are used, the feces and flotation solution mixture can be put through a strainer.
 - 4) Add addition flotation solution to the vial until a slightly convex meniscus is formed at the top.
 - 5) Place a coverslip on top of the vial and wait 10 minutes.
 - 6) After 10 minutes, lift the coverslip from the surface of the vial and place on a clean microscope slide.
 - 7) Examine under 10x and 40x magnification for parasites and parasite ova.

If a laboratory, appropriate equipment and trained personnel are available, the centrifugal flotation methods are the laboratory method of choice. Centrifugal methods are better for detecting some protozoal cysts as well as nematode and cestode eggs. Details for performing centrifugal fecal flotation techniques are available in standard parasitology references and online:

Dr. Micheal Dryden—<http://www.drmichaeldryden.com/parasitology/5/clinical-parasitology>

University of Pennsylvania, Diagnosis of Veterinary Endoparasitic Infections—<http://cal.vet.upenn.edu/projects/dxendopar/index.html>

Other Techniques:

- **Fecal Sedimentation** in water may be necessary to detect trematode eggs. Trematode eggs have a high specific gravity and are difficult to detect by flotation methods. Probably not routinely performed except if other methods of disease surveillance (e.g., necropsy and histopathology) indicate colony health problems with trematodes.
- **Sporulation** in potassium dichromate may be performed to specifically identify oocysts of a coccidian protozoa.

Amphibian Parasites Identified by Fecal Examination

A variety of different parasites, parasite eggs and commensal organisms can be identified on amphibian fecal examination (Poynton and Whitaker 2001). Identification of these eggs is sometimes difficult because few definitive reference sources are available for veterinarians. General characteristics of the eggs from major parasite groups can be found in references relating to reptiles and domestic animals and are helpful in some circumstances (Foreyt 2001; Jacobson 2007; Klingenberg, 2007; Barnard and Upton, 1994). Notes on identification and interpretation of common parasites by fecal examination are detailed below.

Rhabditiform Type Nematodes:

For captive amphibians, these are most important potential findings on fecal parasite screening and include the amphibian lungworms, *Rhabdias* spp. and the intestinal parasites, *Strongyloides* spp.

- *Rhabdias* spp. are common in a wide variety of free-ranging amphibians. In low numbers they do not cause disease.
- Rhabditiforms have a very rapid direct life cycle that leads to superinfections in some captive situations. Husbandry factors such as enclosure hygiene are extremely important to control these parasites.
- Under captive conditions rhabditiform nematodes are identified as a important cause of morbidity and mortality in animals recently brought into captivity for use in survival assurance colonies (Patterson-Kane et al., 2001; Lee et al., 2006; Pessier, 2008; Gagliardo et al., 2008).
- Details on treatment and control of rhabditiform nematodes are reviewed in Section 8.4.

Diagnosis of the rhabditiform nematodes is by observation of characteristic nematode larvae and embryonated nematode eggs on fecal wet mount examination or fecal flotation.

- The eggs are oval, thin-shelled and sometimes referred to as “strongyle-like” or “strongylid-type” and often contain a coiled larval worm (embryonated egg; see Figure 7.6 below)



Figure 7.6: Embryonated eggs typical of a rhabditiform nematode parasite. Each oval egg contains a coiled immature nematode larva. 40X magnification

- Eggs of *Rhabdias* and *Strongyloides* are difficult or impossible to tell apart. If a micrometer is available to measure eggs, the eggs of *Rhabdias* can be larger (approximately 87–120 microns long) than *Strongyloides* sp. (40–55 microns).
- Other non-rhabditiform nematodes of amphibians such as *Cosmocerca*, *Aplectana* and *Oswaldocruzia* have embryonated eggs and might be difficult to distinguish from the rhabditiform nematodes. The sizes of these eggs are closer to those of *Rhabdias* spp. Assistance of a parasitologist and examination of adult worms may be necessary.
- Larvae of *Strongyloides* are unshathed and have a straight tail that appears truncated at the tip. Under oil immersion the tip has a V-shaped notch
- Larvae of *Rhabdias* generally are recognizable by a needle-like stylet on the end of the tail (Figure 7.7). They can be difficult to distinguish from the larvae of free-living nematodes (especially if the sample has been collected from the ground or other area with a lot of organic material).



Figure 7.7: Larva of *Rhabdias* sp. in a fecal wet mount. Note the needle-like stylet on the end of the tail (top of photo).

- The Baerman technique is used to concentrate live rhabditiform nematode larvae. This technique is not often used for routine parasite surveillance, but is useful to obtain large numbers of nematode larvae for identification.
 - Very fresh feces are required.
 - Feces are placed on cheesecloth resting on a wire shelf in a funnel. A tube on the outlet is clamped and funnel filled with warm water (30 °C) to cover the faeces. After six hours water is collected from the tube into a centrifuge tube, spun and examined under a compound microscope for larvae.

Other Gastrointestinal Tract Nematodes:

There are a huge variety of different nematode parasites that are described from the gastrointestinal tracts of amphibians worldwide (Goldberg and Bursery, 2008; Goldberg et al., 2009; Schotthoefer et al., 2009).

- There are very few or no published accounts of these nematodes causing significant disease problems in captive amphibians, but this could occur under

conditions of captive husbandry, especially as more new species are brought into survival assurance colonies.

- There are few descriptions or published photomicrographs to guide clinical diagnosis. As noted above *Cosmocerca*, *Aplectana* and *Oswaldocruzia* (a trichostrongyle) can have embryonated eggs that may need to be distinguished from those of rhabditiform nematodes.
- Oxyurid Type Nematodes (Pinworms). Infections with pinworms are observed in tadpoles (Adamson, 1981) and some postmetamorphic anurans. There is no association with disease and in fact, pinworms may be beneficial commensal organisms (Pryor and Bjorndal, 2005). No treatment is required.

Ciliate Protozoa and Opalinids:

These are common commensal protozoal organisms in the amphibian gastrointestinal tract. They are characterized by cilia (ciliates) or numerous flagella that resemble cilia (opalinids; Poynton and Whitaker, 1994, 2001). The opalinid protozoan *Protoopalina* is shown below in Figure 7.8.

- Species include *Nyctotheroides* (ciliate), *Zelleriella* (opalinid), and *Protoopalina* among others.
- Opalinids are distinguished from ciliates by a lack of a macronucleus or infundibulum.
- Treatment is not required.

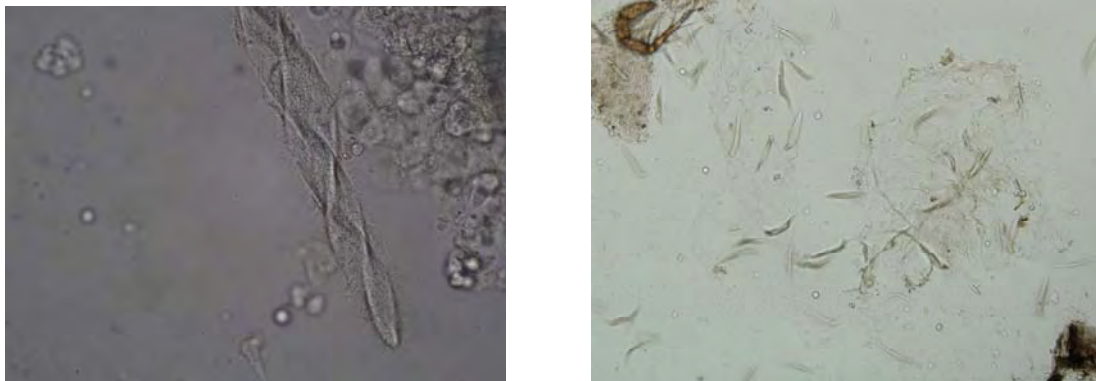


Figure 7.8: The opalinid protozoan *Protoopalina sp.* in fecal wet mount preparations at 10X (right) and 40X magnification (left).

Flagellate Protozoa:

A huge variety of flagellate protozoa are common commensals in the amphibian gastrointestinal tract (Poynton and Whitaker, 2001).

- Flagellates are best appreciated as very small motile organisms on wet mount examination of the feces (see Figure 7.9).
- Treatment is usually not required.

- Stress, recent shipping, or altered environments can lead to flagellate overgrowth and clinical signs (weight loss/failure to thrive). If clinical signs are associated with very high numbers of organisms in a fecal wet mount (5–10 organisms per high power [40x] microscopic field) treatment with medications such as metronidazole can be helpful (Poynton and Whitaker, 2001).

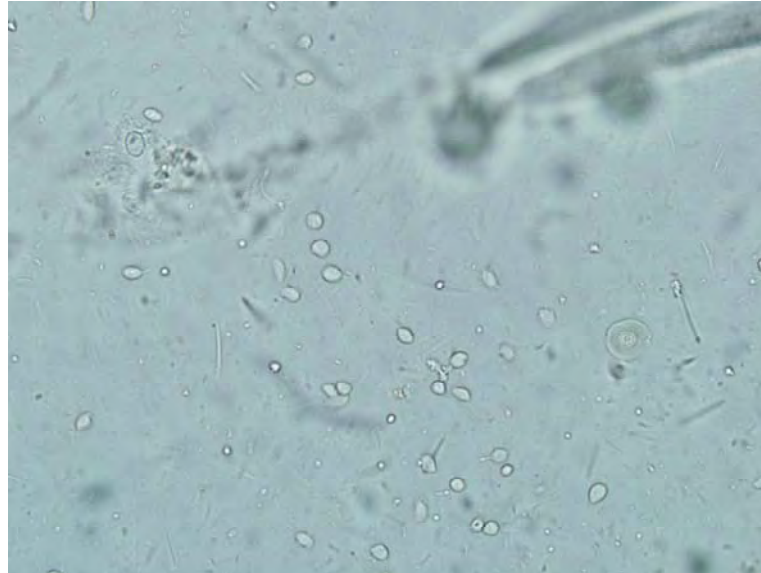


Figure 7.9: Numerous flagellate protozoa in a fecal wet mount from a Kihansi Spray Toad (*Nectophrynoides asperginus*). 40X magnification.

Cestodes (Tapeworms):

Tapeworm eggs are rarely identified on fecal examination (Figure 7.10). Treatment is not required unless weight loss or other clinical signs are observed.



Figure 7.10: Cestode egg in a fecal floatation from an African bullfrog (*Pyxicephalus adspersus*). 40X magnification

Trematodes:

Eggs from trematode parasites or “flukes” (Figure 7.11) are present in the urinary tract (*Gorgoderia* spp.), respiratory tract (*Hematoloechus* spp.) or intestinal tract and are occasionally observed on fecal wet mount examination (Poynton and Whitaker, 2001).



Figure 7.11: Eggs of a trematode parasite (*Hematoloechus* sp.) in a fecal wet mount from a Chiricahua leopard frog (*Lithobates chiricahuaensis*). 40X magnification.

Amoebae:

Amoebiasis caused by the protozoan *Entamoeba ranarum* has very rarely been described as a clinical problem (Valentine and Stoskopf, 1984; Poynton and Whitaker, 2001). Amoebic trophozoites or cysts can be found on fecal wet mounts. Amoebae can be difficult to identify to species, and both pathogenic and non-pathogenic species may be present in fecal samples.

Coccidia:

Protozoal parasites usually of the intestinal tract. Both *Eimeria* and *Isospora* types reported in amphibians.

- Disease problems (coccidiosis) are most often observed in tadpoles or juveniles. Rarely associated with disease in adults.
- Diagnosis is by identification of oocysts on fecal wet mount examination or flotation.
- Treatment may be considered if clinical signs such as weight loss or diarrhea are observed or if necropsy reports on dead animals suggest a heavy parasite burden (Poynton and Whitaker, 2001).

***Cryptosporidium*:**

A protozoal parasite that is only rarely identified in amphibians.

- There is a single case report of disease (proliferative gastritis) in amphibians (Green et al., 2003). Recent description of *C. fragile* from the stomach of toads (Jirků et al., 2008).
- Routine screening is not recommended. If suspected in a group of animals based on necropsy findings, a modified acid-fast stain may be helpful in demonstrating oocysts in feces.

Acanthocephala (Thorny headed worms):

These parasites can be found in the intestinal tract and sometimes associated with clinical problems (intestinal perforation, weight loss) (Poynton and Whitaker, 2001).

7.8 HEMATOLOGY AND SERUM BIOCHEMISTRY

Hematology and serum biochemical analysis are commonly used in health monitoring for mammals, birds and reptiles kept in captive settings. Examination of the cellular fractions of the blood in the form of a white blood cell count is used as an indicator of inflammation (including that associated with infectious diseases). Serum biochemistry analysis can provide information about organ and physiologic function. Hematology and serum biochemistry analysis are only infrequently or rarely performed as part of health assessments and surveillance in amphibians. Reasons for this include:

- Difficulty in collecting adequate volume of blood for desired tests (especially for very small species).
- Lack of standardized normal physiologic ranges for many amphibian species.
- Limited available expertise for review and interpretation of blood parameters including cytologic/morphologic features of amphibian blood.

For these reasons the use of hematology and serum biochemical data for health monitoring of amphibians is in infancy. As interest in amphibian health increases, additional experience is gathered, and prospective research is performed these methodologies may be used with increasing frequency. If data is obtained from amphibian species maintained in survival assurance populations efforts should be made to carefully record the laboratory methods used to compile this information as well as the numerical data. This is because the choice of laboratory method influences the results and the ability to compare findings between laboratories depends on this information.

Sample Collection

There are several recent reviews of amphibian blood collection techniques and hematology (Wright, 2001a; Campbell and Ellis 2007; Allender and Fry, 2008; Heatley and Johnson, 2009).

- Common venipuncture sites include the midline abdominal vein (anurans); lingual plexus; caudal tail vein (salamanders) and cardiac puncture.

- Heparin is the preferred anticoagulant. EDTA is associated with red blood cell lysis in many species.
- Dependent upon the size of the specimen, it is safe to collect up to 1 % of body weight (e.g., 0.1ml blood per 10g body weight) from a healthy animal; smaller volumes should be collected from animals that are sick or debilitated.
- Serum or plasma can be colored blue, green or orange, even in healthy animals.
- If an experienced veterinarian is available, use of tricaine methanesulfonate (MS-222) or clove oil anesthesia can make blood collection easier with reduced stress (Wright 2001b; Mitchell, 2009).

Sample Analysis

- For very small animals information can be gained by performing an estimate of the white blood cell count on a stained blood smear.
- Complete blood counts are performed using manual methods such as those that use a Natt-Herricks solution.
- Morphology and function of amphibian white blood cells is an area that requires additional research. There are morphologic forms of granulocytes that resemble neutrophils and eosinophils (in contrast to the “heterophils” of birds and reptiles).
- Commercial veterinary diagnostic laboratories that accept samples from reptiles can be used to analyze samples from amphibians. If the laboratory has pre-existing panels for reptile species, these can be used for amphibians, however, one suggested substitution is to include blood urea nitrogen instead of uric acid (reflecting the predominant amphibian nitrogenous waste products) except in the rare uricotelic amphibians (e.g., *Phyllomedusa* spp. and *Chiromantis* spp.)
- Interpretation of results can be difficult. A variety of factors including hydration status, seasonality and sex influence hematological and biochemical parameters to a greater degree than other species commonly encountered by zoo veterinarians.
- Few normal reference values are available. A summary is available (Wright 2001a). The International Species Information System databases may be a source of additional information (www.isis.org/CMSHOME/)
- For interpretation of results from a sick animal, it is useful to also obtain a sample for comparison from a normal animal of the same species, sex and housing conditions.

7.9 LABORATORIES THAT ACCEPT AMPHIBIAN SAMPLES

Necropsy and Histopathology

United States

- Northwest Zoopath, 654 W. Main St., Monroe, WA 98272
Phone: (360) 794-0630 Fax: (360) 794-4312 (zoopath@aol.com)

- Zoo/Exotic Pathology Service, 2825 KOVR Drive, West Sacramento, CA 95605
Phone: (916) 725-5100 (Mail@zooexotic.com)
- Amphibian Disease Laboratory, San Diego Zoo's Institute for Conservation Research
(apessier@sandiegozoo.org)

Molecular Diagnostic Testing ("PCR")

United States

- Pisces Molecular. Conventional PCR for amphibian chytrid fungus *Batrachochytrium dendrobatidis*. (www.pisces-molecular.com)
- Amphibian Disease Laboratory, San Diego Zoo's Institute for Conservation Research. Taqman PCR for amphibian chytrid fungus and ranaviruses. (www.sandiegozoo.org/conservation/news/low-cost_health_screening_for_captive_amphibians)
- School of Biological Sciences, Center for Integrated Biotechnology, Washington State University. Taqman PCR for amphibian chytrid fungus and ranaviruses. (astorfer@wsu.edu)
- Zoologix Laboratories. Taqman PCR for amphibian chytrid fungus *Batrachochytrium dendrobatidis* and ranaviruses. (www.zoologix.com)
- Research Associates Laboratories. www.vetdna.com
- University of South Dakota, Biology Department. Jacob.Kerby@usd.edu

United Kingdom

- Zoological Society of London. matthew.perkins@ioz.ac.uk

Australia

- Australian Animal Health Laboratory, Geelong. OIE diagnostic reference laboratory for chytridiomycosis and ranaviruses (Alex.Hyatt@csiro.au).
- James Cook University, Townsville. Diagnostic research laboratory for chytridiomycosis (lee.skerratt@jcu.edu.au).

Switzerland

- Ecogenics Laboratory. www.ecogenics.ch/indexhtml

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CHAPTER 8

DISEASE TREATMENT AND CONTROL

8.0 INTRODUCTION

Among the most important infectious disease issues identified in amphibian survival assurance populations are chytridiomycosis, *Ranavirus* infection, and infection with the rhabditiform nematodes *Rhabdias* and *Strongyloides*. The goals of the medical treatment and other disease control measures described in this chapter are to:

- Mitigate the effects of infectious diseases on the success and sustainability of captive amphibian populations.
- To reduce the risk that captive amphibian populations could serve as sources of population limiting infectious diseases (e.g., chytridiomycosis and *Ranavirus*) for wild amphibian populations. These captive populations can be defined as not only amphibians in survival assurance populations, but also those with other roles such as animals for display and education and animals that are used for commercial purposes (e.g., pet trade, laboratory animals or food).
- Identify methods that can be used to create specific pathogen free amphibian populations, especially for those types of amphibians that are frequently moved as the result of amphibian trade or conservation programs.

It should be clearly recognized by veterinarians and others that implement treatment regimes for infectious diseases that very few therapeutic recommendations for amphibians are based on blinded, controlled experimental trials. Most treatments have been empirically derived and therefore are not efficacious in all circumstances or may not be safe for use on all amphibian species or life-stages. Finally, for some pathogens (e.g., *Ranavirus*) specific treatment methods are not available and the emphasis will be on other disease control methods.

For survival assurance populations a decision to treat animals for a specific infectious disease or the goals of treatment (e.g., complete elimination of a parasite by treatment or treatment to reduce parasite numbers but not eliminate infection) will depend on factors such as:

- The significance of the pathogen to the health of the captive population. If minimal health effects are seen in the captive animals treatment may not be necessary.
- It may be desirable to maintain survival assurance populations that are infected with pathogens or parasites that are naturally found in the wild population. Presumably, this allows captive animals that will later be returned to the wild to maintain host species adaptability or immunity to a specific pathogen.

- It may be desirable to develop survival assurance populations that have acquired some resistance or exposure to pathogens (e.g., amphibian chytrid fungus) that are responsible for the declines of the wild population.
- Decisions to treat an individual or population for a pathogen will also depend upon the occurrence of the disease in conspecific and contact animals, other captive populations, and in wild populations.

8.1 TREATMENT AND CONTROL METHODS FOR THE AMPHIBIAN CHYTRID FUNGUS

Treatment methods for the amphibian chytrid fungus are necessary for:

- Reducing morbidity and mortality in captive populations due to this infection.
- Salvage of wild amphibian populations experiencing mortality due to chytridiomycosis (Gagliardo et al., 2008).
- Reducing risk posed to both captive and wild amphibian populations by amphibians that are infected with the chytrid fungus and can introduce the fungus to new locations.
- Creation of breeding populations of amphibians that are known to be free of chytrid fungal infection (specific pathogen free). These populations can be used as survival assurance populations or for commercial purposes (e.g., food, laboratory animals and pets).

The amphibian chytrid fungus has very low host-specificity as demonstrated by identification in over 200 species and 20 families representing both anurans and caudates. While a wide range of amphibian species (probably most amphibian species) can become infected, different amphibians vary considerably in their susceptibility to the disease caused by Bd (chytridiomycosis). For many species the infection does not cause outward signs of disease (subclinical infection), but these infected animals can still act as sources of infection for species that are highly susceptible to the disease caused by Bd (chytridiomycosis). For this reason it is usually necessary to treat captive amphibians for infection when it is identified either as a cause of disease or when a subclinical infections are identified using PCR testing (see Sections 6.7–6.8 and Chapter 8).

Although successful treatment for amphibian chytrid fungus has been accomplished, there are disadvantages of all of the currently described treatment methods (Young et al., 2007; Berger et al., 2010). These disadvantages include:

- Treatments are not consistently successful across species in eliminating chytrid infection.
- There is a need for controlled experimental trials of treatment methods for chytrid infection. Most treatments have been empirically-derived and tested on a single species or only a small number of individuals. Trials that look at a variety of different amphibian species and that evaluate treatment efficacy as

well as tolerance and safety between species are desperately needed. Other trials may examine combinations of different treatments (e.g., combination of heat and antifungal drug).

- Some treatment medications or treatment regimes are toxic or are not tolerated by some amphibian species or specific amphibian life-stages (e.g., tadpole). Many reports of treatment-associated deaths are poorly documented
- Some treatments have a potential risk to human health (e.g., malachite green; chloramphenicol).
- There are few reliable treatments for tadpoles (Garner et al., 2009).

Some general comments about all treatment methods for the amphibian chytrid fungus are:

- There are significant species and life-stage (e.g., tadpole, juvenile, and adult) differences in the ability to tolerate antifungal medication. If there is no experience with a specific medication in a species or life-stage it is advisable to first treat a small number of individuals to evaluate safety before treating a whole group of animals.
- Because the infection is located in a superficial location on the skin treatments are usually applied topically as a bath. It is important to periodically agitate the treatment solution to ensure contact of the medication with all skin surfaces (dorsal and ventral).
- Because the chytrid fungus persists in the environment it is important that animals are placed into a chytrid-fungus free enclosure after every daily treatment. See step # 1 in the itraconazole treatment protocol given below. Daily disinfection of enclosures and enclosure furniture is necessary (see Chapter 5).
- Animals should not be returned to a contaminated permanent enclosure after treatment for the chytrid fungus. Permanent enclosures need to be thoroughly cleaned and disinfected. New substrates should be used.
- Because treatments are not uniformly effective, the use of post-treatment PCR testing to evaluate animals for treatment success or failure is strongly recommended. Multiple tests may be required to be confident that animals are free of infection after treatment (see Section 7.3).
- If PCR testing is positive after the first treatment course, a second treatment course with the same or different medication or method is necessary to ensure clearance of Bd infection. Sometimes, multiple cycles of treatment and testing are needed to ensure clearance of infection.
- If animals are sick or dying with Bd infection (chytridiomycosis) it is helpful to provide supportive care with supplemental electrolytes (Voyles et al. 2007; Voyles et al., 2009; Berger et al., 2010) and also antibiotics (e.g., Baytril). See Section 8.5 for details on formulating electrolyte supplementation solutions.

- As institutions begin to broadly diagnose and treat Bd in their collections, an opportunity exists to anecdotally determine which treatment protocols prove most efficacious for which species. Collection of information including species, body size, dose used, dosing methodology, treatment period, follow-up diagnostics, results, and need for repeat treatments are helpful. It is as important to collect data on what does not work as well as what does work.

A variety of treatment methods have been described in the literature or anecdotally within amphibian conservation programs. These have been recently reviewed (Berger et al., 2010). The type of treatment selected will depend on several factors including:

- Cost.
- The availability of specific medications.
- The number of animals that must be treated.
- The known or anticipated tolerance of the species for treatments such as elevated environmental temperature or itraconazole.

Provided below is information regarding the transmission of the amphibian chytrid fungus and specifics on some of the most commonly used treatment methods.

Transmission of the Amphibian Chytrid Fungus

Aspects of the transmission of the amphibian chytrid fungus that are helpful in designing a treatment and control program include:

- The infective stage of the amphibian chytrid fungus responsible for transmission of infection is the flagellated zoospore. The occurrence of resistant spore or resting stage has not been clearly documented.
- It is possible to house amphibians infected with the chytrid fungus in the same room or facility as non-infected amphibians without transmission of infection. This requires careful attention to biosecurity practices (see below).
- Infective zoospores are very susceptible to desiccation (complete drying) therefore transmission usually requires moist or wet materials and tools or animal-to-animal contact (Johnson and Speare, 2003; Piotrowski et al., 2004)
- The chytrid fungus has been shown to survive in deionized water for 3–4 weeks, sterilized lake water for 7 weeks, moist river sand for 3 months, and bird feathers for up to 3 hours (Johnson and Speare, 2003; Johnson and Speare, 2005).
- Biosecurity practices that are helpful for controlling infection and transmission are:
 - Avoid the transfer of animals, moist or wet substrates (e.g., soil, gravel, moss, plants), cage furniture or water between different enclosures. Disinfect tools and equipment between uses in different enclosures.

- Follow recommendations for hygiene, work-flow patterns and enclosure sanitation in Sections 4.9–4.12 and Chapter 5.
- Use disinfection practices for enclosures and equipment that are known to kill the amphibian chytrid fungus. See Chapter 5.

Treatment with Itraconazole

The azole-type antifungal drug itraconazole is commonly used in zoos and amphibian conservation programs for treatment of chytridiomycosis in postmetamorphic amphibians (Nichols et al., 2000; Forzan et al., 2008; Gagliardo et al., 2008; Pessier, 2008). There are some controlled clinical trials to support the use of itraconazole, however, these trials have been based on small numbers of animals or just a small number of species (Nichols et al., 2000; Lamirande and Nichols, 2001; Garner et al., 2009).

Advantages of itraconazole include:

- Successful use in several amphibian conservation programs. In the United States captive breeding programs for the Wyoming toad (*Anaxyrus baxteri*) and the Puerto Rican crested toad (*Peltophryne lemur*) have used this medication extensively. Use of itraconazole in a rescue operation of a variety of Panamanian amphibian species appeared to be well-tolerated (Gagliardo et al., 2008).
- Itraconazole may have an advantage over other azole antifungal drugs because in mammals it becomes concentrated and persists in keratinized tissues such as the skin. This has not been proven for amphibians.
- The daily treatment application is for a short period of time (5–10 minutes).
- Fewer human safety concerns compared to some other described treatment methods.

Disadvantages of itraconazole are:

- The treatment protocol that has been most frequently used for post-metamorphic amphibians in captive amphibian programs is toxic to tadpoles and some recently metamorphosed frogs. This standard protocol using a 0.01% itraconazole solution should not be used in these life-stages. An alternative protocol using a substantially lower concentration of itraconazole was tolerated by *Alytes muletensis* tadpoles, but resulted in skin depigmentation (Garner et al., 2009).
- There is variation in how postmetamorphic animals tolerate treatment with the standard 0.01% itraconazole solution. There have been anecdotal reports of treatment-associated anorexia, corneal ulcers, kidney disease and deaths in some species (especially ranid frogs). When working with a novel species, or life-stages, treatment should be tested and evaluated carefully before widespread application.

- Observations of adverse effects have not been consistent and the same species may tolerate treatment well at one facility and have a negative treatment outcome at a different facility. Factors influencing treatment outcome could include severity of disease prior to treatment; variation in how treatment medication is formulated or applied; and idiosyncratic drug reactions.
- The cause of treatment-associated deaths could also include factors related to the formulation of itraconazole. To achieve solubility of the drug in an aqueous form the commercial oral solution is very acidic. Low pH may result in skin irritation or osmotic dysfunction and this may be difficult to tolerate for animals that are already osmotically compromised because of disease or for totally aquatic forms such as tadpoles. Use of less concentrated itraconazole solutions or application of treatment in solutions with buffering capacity such as amphibian Ringer’s solution may be helpful.
- Some of the azole-type antifungal drugs (like itraconazole) decrease synthesis of steroid hormones such as testosterone or corticosteroids which could impact reproductive viability. For itraconazole these side effects are minimal in mammals, but effects in amphibians have not been studied. Some amphibians undergoing itraconazole treatment have subsequently successfully reproduced in assurance populations (Wyoming Toad, Panamanian Golden Frog, Puerto Rican Crested Toad).
- The commercially available itraconazole oral solution is expensive and treatment of large numbers of animals may be cost-prohibitive.



Figure 8.1—A hyloid frog (*Hyloscirtus colymba*) from Panama showing symptoms of a clinical case of chytridiomycosis that was contracted in the wild. Photo by E. Baitchman.

Itraconazole Treatment Protocol

The itraconazole treatment protocol most often used in amphibian conservation programs was developed in small trials with captive dendrobatid frogs at the

Smithsonian National Zoo (Nichols et al., 2000; Lamirande and Nichols, 2002). The protocol was empirically derived and subsequently applied to a variety of amphibian species in captive settings. Uses have included treatment of animals sick with chytridiomycosis, treatment of animals subclinically infected with the amphibian chytrid fungus and for prophylactic treatment of high risk animals prior to hibernation, shipment, breeding, translocation or reintroduction to the wild.

The original protocol used a compounded suspension of itraconazole diluted in 0.6 % NaCl (saline) daily for 11 days. Subsequently, many variations of the protocol have emerged and most use a commercially available 10mg/ml (1%) oral solution of itraconazole (Sporanox[®] Oral Solution; Itrafungol[®] Oral Solution) to make a 0.01% treatment solution by dilution in amphibian Ringer's solution.

The following protocol is recommended:

1. During the treatment period animals are kept in enclosures that are easy to disinfect. Options include temporary enclosures made from plastic food storage containers ("Tupperware[®]"), inexpensive plastic animal enclosures ("Pet Pals[®]"), glass aquariums, and large plastic storage containers ("Rubbermaid[®]").
 - Temporary enclosure substrates should be disposable (e.g., paper towels, moist sphagnum moss) and changed daily.
 - Temporary enclosure cage furniture (e.g., hide boxes) should be made of easily cleaned and disinfected material. These should be disinfected daily.
 - It is helpful to have 2 sets of enclosures and cage furniture that are alternated between days of treatment. After animals receive treatment they are placed into the clean enclosure that had been disinfected the previous day (see below).
2. If there is no previous experience using itraconazole in the species or life-stage (e.g., tadpole, juvenile, or adult) that is being treated, the treatment protocol should be tried on a small number of animals to evaluate safety before treating a large group of animals. This may not always be possible in situations with sick and dying animals. Consider treating these animals with lower concentrations of itraconazole (see # 3 below).
3. The itraconazole treatment solution is prepared fresh daily. The commercially available 10mg/ml (1%) oral solution of itraconazole (Sporanox[®] Oral Solution; Itrafungol[®] Oral Solution) is diluted in amphibian Ringer's solution (see Section 8.5 for recipe) to make an 0.01% treatment solution. Do not use the 0.01% treatment solution on tadpoles or very recently metamorphosed animals (lower concentrations of itraconazole may be safe, see below).

- To make each 100 ml of treatment solution, add 1.0 ml of 10mg/ml itraconazole to 99 ml of amphibian Ringer's solution (to make 1 liter of treatment solution add 10ml of 10mg/ml itraconazole to 990 ml of amphibian Ringer's). This is equivalent to 100mg of itraconazole per liter of treatment solution.
 - Recent experience shows that use of itraconazole concentrations less than 0.01% can be successful at eliminating chytrid fungal infection. Experimental trials to determine the minimum effective concentration of itraconazole are needed. The use of lower concentrations is suggested for treating species that do not tolerate treatment with the 0.01% solution or if there is no prior experience using itraconazole in a species. Some workshop attendees have started to use 0.005% (50 mg per liter) treatment solutions or less with success.
 - A single experimental trial successfully treated tadpoles of the Midwife Toad (*Alytes muletensis*) with a very low concentration of itraconazole (0.5–1.5 mg itraconazole per liter of treatment solution). However, these tadpoles lost skin pigmentation and therefore the long-term safety of this treatment for tadpoles is unknown (Garner et al., 2009). This very low dose is not suggested for post-metamorphic animals at this time.
4. The itraconazole bath treatment is applied for 5 minutes once daily for 10 consecutive days.
- The use of plastic bags with a zipper-type closure (e.g., Ziploc®), disposable plastic cups (Forzan et al., 2008) or disposable plastic food containers are helpful for the application of the itraconazole baths. The small volume required for these containers reduces the amount of treatment solution needed. The use of the plastic bags has appeared to reduce the stress of treatment for some animals.
 - The itraconazole solution will turn white or “milky” when added to the amphibian Ringer's solution. Some animals will react when placed in the treatment solution and try to escape from the bath (this may reflect skin irritation). If extreme reactions are noticed use of a lower concentration of itraconazole solution is considered.
 - The treatment solution should cover the ventral skin surfaces and extend approximately half way onto the lateral body surfaces. Animals should not swim or float in the treatment solution (except for totally aquatic amphibian species).
 - The treatment container is periodically agitated to ensure that the treatment solution reaches all skin surfaces (dorsal and ventral).

Discourage animals from climbing onto the sides of the treatment container to escape the treatment solution.

- After each daily treatment animals are returned to a clean and previously disinfected enclosure (see # 1 above).
5. For animals that are clinically ill with chytridiomycosis (e.g., showing signs of lethargy, anorexia, poor righting reflexes, excessive skin shedding, hunched posture) supportive treatment with supplemental electrolytes and antibiotics can be helpful.
- Experimentally infected frogs with terminal chytridiomycosis were shown to be hyponatremic and hypokalemic (Voyles et al., 2007; Voyles et al., 2009).
 - To attempt to correct electrolyte abnormalities, oral 12% Whitaker-Wright solution administered by stomach tube has been suggested (Voyles et al., 2009; Berger et al., 2010). See Section 8.5 for the Whitaker-Wright formulation.
 - Alternatively or in addition, animals that are clinically affected by chytridiomycosis are placed in an amphibian Ringer's bath prepared at isotonic or slightly greater than isotonic concentration, in order to encourage retention of electrolytes. The normal water source can be replaced with amphibian Ringer's. The solution is changed daily.
 - Empirical antibiotic treatment (e.g., enrofloxacin) is also administered to treat secondary bacterial infections.
 - A review of amphibian fluid therapy is found in Wright and Whitaker (2001). Formulas for electrolyte solutions are given in Section 8.5.
6. Treatment is discontinued after 10 days. The success of treatment can be verified by PCR testing of skin swabs (See Sections 7.2–7.3).
- Samples for PCR testing are obtained 2 weeks after the end of treatment. This allows animals to finish shedding skin that might contain inactivated or dead chytrid organisms.
 - Because treated animals may have very low levels of infection it is suggested that at least 2 to 3 PCR swabs be obtained over a 2 week period. Multiple negative PCR tests allow for greater confidence that animals have been successfully cleared of infection
 - Pooling of samples from multiple animals in the same treatment group can help to reduce costs of post-treatment testing. If any animals in the treatment group test positive, the entire group is considered infected and re-treated.

7. A single 10 day treatment cycle is not always effective in eliminating infection with the amphibian chytrid fungus. A second treatment cycle and occasionally multiple treatment cycles might be required to clear animals of infection. Some possible causes of treatment failure include:
- Failure to disinfect animal enclosures after each daily treatment application.
 - Failure to agitate the treatment solution to ensure that all skin surfaces are coated with medication or allowing animals to escape from the treatment solution (e.g., climbing on the side of the treatment container).
 - If compounded itraconazole suspensions are used instead of the commercially-available 10mg/ml oral solution, the itraconazole may come out of solution and settle on the bottom of the treatment container. Frequent agitation and mixing may be required for treatment solutions made from itraconazole suspensions.
8. Field application of the itraconazole treatment protocol can easily be done and can increase survival of animals from a Bd-positive environment that are being collected for conservation assurance colonies. Animals that are Bd-positive when captured may rapidly develop clinical illness due to stress-induced immunosuppression. Those animals that are captured on the first days of a multi-day expedition may especially be at risk of developing irreversible disease by the time all are brought back to the primary quarantine and treatment facility. In known Bd-positive regions, beginning the treatment protocol within the first 24 hrs of capture can markedly increase overall survival rates.
- Sealable disposable plastic bags, disposable plastic cups, and itraconazole stock solution are easily carried in to the field. Local water sources may be used to prepare the diluted treatment solution by using portable water filtration devices that include 0.5 micron filters or smaller, to remove Bd organisms from the water source. Portable water filtration devices made for producing potable water for hikers are readily available at outdoor supply retailers.
 - Carrying amphibian Ringer's stock solution and antibiotics in to the field will also allow supportive treatment of animals that are found already clinically ill in heavily affected areas.

Treatment with Chloramphenicol and Florfenicol

The antibiotic chloramphenicol has paradoxically been shown to have both *in-vitro* and *in-vivo* activity against Bd (Poulter et al., 2007; Bishop et al., 2009). Initial trials using a continuous bath of chloramphenicol for 2–4 weeks was apparently safe and effective in eliminating Bd infection in Southern Bell Frogs (*Litoria raniformis*) and Brown tree Frogs (*Litoria ewingii*).

Potential advantages of treatment with chloramphenicol are:

- Treatment appears to be well-tolerated by both tadpoles and adult frogs even when animals are kept continuously in treatment solution for several weeks.
- Treatment may be one of the few safe medications available for use in tadpoles.
- Application by continuous immersion is very convenient for treatment of totally aquatic amphibian species and tadpoles.
- Treatment is inexpensive compared to itraconazole.

However, there are some potentially significant disadvantages to the use of chloramphenicol:

- The available treatment protocols require that animals be continuously exposed to the treatment solution for 2–4 weeks. This can be tolerated by many aquatic or semi-aquatic amphibian species, but terrestrial amphibian species (e.g., toads) could have problems with osmoregulation and fluid balance under these conditions. Experimental trials with terrestrial amphibian species are needed before placing these animals into an extended bath treatment protocol.
- Chloramphenicol is very rarely associated with bone marrow suppression and aplastic anemia in cats and human beings. This may raise occupational health regulatory concerns in some countries. Workers treating amphibians with chloramphenicol should use precautions to avoid exposure to the treatment solution. The use of drugs such as florfenicol which are related to chloramphenicol but do not have human health concerns should be evaluated experimentally for use in the treatment of chytridiomycosis.

A detailed treatment protocol for the use of chloramphenicol is available online at: [www.nzfrogs.org/site/nzfrog/files/Treatment Protocol.pdf](http://www.nzfrogs.org/site/nzfrog/files/Treatment%20Protocol.pdf)

- The treatment solution is made from reagent grade chloramphenicol (chloramphenicol C0378; Sigma-Adrich, St. Louis, MO). A stock solution is made by adding 200 mg of the chloramphenicol powder to 1 liter of hot water. One part of the stock solution is diluted in 9 parts water to make the treatment solution (e.g., 100ml of stock solution added to 900 ml of water to make 1 liter of treatment solution). The treatment solution contains 20mg per liter (20ppm) of chloramphenicol.
- Animals are placed into the treatment solution for 2–4 weeks and must have constant exposure to the treatment solution. The treatment solution is changed daily.
- Preliminary attempts to treat animals with florfenicol (NuFlor®) using a treatment solution of 30 mg per liter (30ppm) has been tolerated by some amphibian species (K. Wright, personal communication). Studies are needed to

confirm efficacy against the amphibian chytrid fungus. It is also unknown if there are additives in the commercially available florfenicol injectable solution (marketed for cattle in the United States) that may be toxic to amphibians. Use of florfenicol powder obtained from a compounding pharmacy is preferable.

- At the conclusion of treatment PCR testing as described above in the itraconazole treatment protocol is suggested to confirm elimination of infection.

Elevated Environmental Temperature

The use of elevated environmental temperature to treat infection with the amphibian chytrid fungus exploits the inability of the fungus to grow at higher temperatures (maximal growth at 17–25°C). In a pilot study, juvenile Green Tree Frogs *Litoria chloris* experimentally infected with the chytrid fungus and held for 16 hours at 37°C were cleared of infection while most animals held at 20°C died of chytridiomycosis (Woodhams et al., 2003). Use of 32°C by continuous exposure was reportedly effective in clearing Western Chorus Frogs *Pseudacris triseriata* of infection (Retallick and Miera, 2007).

- As with other treatment methods temperature elevation has been inconsistently effective between species. It is not recommended as the sole method of treatment in most cases. Use of temperature elevations in combination with another treatment method (e.g., itraconazole or chloramphenicol) is suggested.
- Not all amphibian species can tolerate the higher environmental temperatures (37°C) needed for rapid elimination of infection by application of heat. However, use of lower temperatures that still exceed the ideal growth temperatures of the fungus might still aid in clearing of infection. For instance, *Mixophyes fasciolatus* inoculated with the chytrid fungus and housed at 27°C had no evidence of infection by 98 days post-innoculation (Berger et al., 2004).
- When considering temperature as a treatment method for Bd, it is important to maintain animals at a constant rather than intermittent temperature elevation. (Young et al., 2007).
- Additional experimental trials using heat to eliminate infection with the amphibian chytrid fungus are needed. Heat treatment may be useful in combination with other treatment methods.

Other Treatment Methods

Several other treatment methods are described or are used anecdotally. These have been recently reviewed (Berger et al., 2010). Some of these methods have significant disadvantages.

- **Malachite green and formalin.** This is a combination of chemicals that has been used extensively as an antiprotozoal and antifungal bath for fish. Routine use to treat infection with the amphibian chytrid fungus is not recommended.

- A combination of 0.1mg/liter malachite green and 25 ppm formalin administered as a bath for 24 hours every other day for a total of 4 treatments was effective at treating African Clawed Frogs (*Xenopus tropicalis*) (Parker et al., 2002).
- Although this treatment protocol could be considered for use in other species, both malachite green and formalin are known to be teratogenic and/or carcinogenic and are associated with significant human health concerns.
- Many amphibians will not tolerate treatment with these chemicals.
- **Benzalkonium chloride.** Benzalkonium chloride is a quaternary ammonium disinfectant occasionally used as an antifungal medication in fish and amphibians. It has been mentioned as a potential treatment for chytridiomycosis in the pet trade: www.flippersandfins.net/chytridBCTreatment.htm However, use of benzalkonium in dwarf African clawed frogs with chytridiomycosis (originally diagnosed as infection with *Basidiobolus ranarum*; see Groff et al., 1991) resulted in reduced numbers of deaths, but did not eliminate infection with the chytrid fungus. Benzalkonium is not suggested as a definitive treatment at this time.
- **Azole antifungal medications other than itraconazole.** Protocols using miconazole and fluconazole have been described (Nichols et al., 2000; Berger et al., 2010), but are not in wide use or have had marginal efficacy.
- **Trimethoprim-sulfadiazine.** This combination appeared to have some fungistatic activity in a limited trial with dendrobatid frogs (Nichols et al., 2000).
- **Terbinafine (Lamisil®).** This is an over-the-counter antifungal medication that many private hobbyists and pet owners have used to treat animals suspected to be carrying or to be clinically ill with chytridiomycosis. Protocols are readily found on line, though there have been no controlled studies to confirm efficacy or safety of this treatment and in most cases, Bd has not been confirmed in animals prior to treatment. This protocol is not recommended.

8.2 CONTROL OF RANAVIRUS INFECTIONS

Infections with ranaviruses have only recently been recognized as a potential problem in captive amphibian populations (Miller et al., 2008; Pasmans et al., 2008; Driskell et al., 2009) and the extent and significance of these infections is unknown. Greater efforts to survey for and diagnose *Ranavirus* infections in captive populations are necessary in order to fill in these knowledge gaps.

Treatment options for viral pathogens are very limited in vertebrates in general and are of unknown efficacy in amphibians. If *Ranavirus* infections are diagnosed in a captive amphibian population most efforts will focus more on disease control rather than treatment of individual animals. The goals of these control measures are to:

- Avoid transmission of infection to other amphibians in the population or facility.

- Avoid transmission of infection to native amphibian populations.
- Creation of breeding populations of amphibians that are known to be free of ranaviral infection (specific pathogen free). These populations can be used as survival assurance populations or for commercial purposes (e.g., food, laboratory animals and pets).

Subclinical infections with ranaviruses have been documented with periods of persistence ranging from as little as 20 days to six months or more (Brunner et al., 2004; Robert et al., 2007).

- Subclinically infected animals have the potential to inadvertently spread infection to other, more susceptible, animals. However, this has not been documented in captive populations.
- Unfortunately, unlike infection with the amphibian chytrid fungus, reliable diagnostic tests to detect animals subclinically infected with ranaviruses are not yet available (see Section 7.4) and this complicates implementation of disease control measures.

Transmission of *Ranavirus* Infection

Aspects of *Ranavirus* transmission that are helpful in designing a treatment and control program include:

- Transmission occurs by routes such as direct animal contact, exposure to water previously containing infected animals, consumption of infected animal tissues and potentially by contaminated tools, equipment and enclosures.
- The EHN *Ranavirus* remains viable for greater than 97 days in cell-free distilled water held at 15°C (59°F) and for greater than 113 days on dry surfaces (Langdon, 1989).
- The *Ambystoma tigrinum-Ranavirus* can remain viable in water for up to 2 weeks at 25°C (77°F; Jancovich et al., 1997).

Treatment and Control Methods

If an outbreak of ranaviral disease is identified in a captive population control methods that are useful include:

1. Isolation of sick animals from healthy animals.
2. Strict adherence to biosecurity practices that minimize or eliminate transmission of pathogens between animal enclosures. This can minimize the number of animals that become sick or die during an outbreak. Practices to control infection and transmission are:
 - Avoid the transfer of soiled substrates (e.g., soil, gravel, moss, plants), cage furniture or water between different enclosures. Recommendations

for hygiene, work-flow patterns and enclosure sanitation are detailed in Sections 4.9–4.12 and Chapter 5.

- Disinfect tools and equipment between use in different enclosures. Disinfectants known to inactivate ranaviruses are listed in Chapter 5.
3. There are limited options helpful for treating individual animals with *Ranavirus* infection.
 - In Tiger Salamanders (*Ambystoma tigrinum*) exposed to the ATV *Ranavirus*, environmental temperatures influenced mortality and time to death. Most animals survived at 26°C (78.8°F) and most died at 18°C (64.4°F) and 10°C (50°F) (Rojas et al., 2005).
 - Treatment with antibiotics (e.g., enrofloxacin) could help control secondary bacterial infections.
 4. A risk assessment should be performed for animals that are known to be infected with a *Ranavirus* or that have survived a *Ranavirus* outbreak. This is discussed in more detail in Section 6.2. Briefly, decisions about the management of these animals will depend on:
 - The importance of the infected or exposed animals to the captive population and to species recovery efforts.
 - The results of disease surveillance efforts for *Ranavirus* infection. The best samples for surveillance are tissue samples collected at the time of necropsy examination. There are few reliable or validated tests for *Ranavirus* infection in living animals.
 - The presence of the same *Ranavirus* infection in the captive population as exists in the wild population of a particular amphibian species. This requires specialized techniques such as RFLP analysis that is beyond the standard PCR-based tests. See Section 7.4

8.3 CREATING SPECIFIC-PATHOGEN-FREE AMPHIBIAN POPULATIONS (AMPHIBIAN CHYTRID FUNGUS AND RANAVIRUS)

Captive animal populations free of specific important pathogens (“specific-pathogen-free” or SPF) have been created in agricultural settings, aquaculture, and laboratory animal colonies and similar approaches could also be very useful in the management of captive amphibian populations (Lotz, 1997; OIE Aquatic Health Code: http://www.oie.int/Eng/normes/fcode/A_summry.htm). As noted in Chapter 4, there are major concerns about the movement and introduction of amphibian pathogens to new geographic locations and the impact of introduced pathogens to wild amphibian populations.

Creation of SPF amphibian populations for amphibians commonly distributed for the pet trade (e.g., dwarf African Clawed Frogs; White’s Tree Frogs); laboratory research (African Clawed Frogs; Leopard Frogs); food production (American Bullfrogs) and amphibian survival-assurance populations is strongly encouraged in order to:

- Reduce of the risk of moving important amphibian pathogens such as the chytrid fungus or ranaviruses to new locations by means of amphibian trade.
- Reduce the need for extensive infectious disease testing prior to shipment or during quarantine (see Chapter 6) if animals are known or certified to be free of specific pathogens. This results in:
 - Reduced time in quarantine and reduced animal stress associated with disease testing.
 - Reduction in disease testing costs.
 - May allow for easier compliance with World Organization for Animal Health (OIE) requirements for amphibian movements (see Section 7.6)
- Reduce the impact of specific infectious diseases on the sustainability of captive populations and on the success of survival assurance populations.

Potential challenges of creating SPF populations are:

- Populations are expensive and time-consuming to create.
- Maintenance of the SPF status in a population and prevention of re-infection require a long-term commitment to:
 - Maintaining strict facility biosecurity practices (see Chapter 4). Some of the more important biosecurity practices are preventing exposure to wild amphibians or to cosmopolitan amphibian collections that keep animals from multiple geographic locations or sources.
 - Keeping careful quarantine practices in effect, before new animals are introduced to a population (see Chapter 6).
 - Creating and using a disease surveillance program that includes necropsy and histopathology of animals that die (see Chapter 9) as well as periodic specific testing for pathogens of interest.
- In some cases it may be desirable to maintain infection with specific pathogens at levels that do not result in significant morbidity and mortality within a captive population. This is most applicable to survival assurance populations where it is desirable that captive animals develop or maintain tolerance to pathogens they will encounter when reintroduced to the wild.

In general, creation of SPF populations requires:

- The availability of a reliable diagnostic test or tests for the pathogen of interest.
- The availability of specific disease treatments effective in eliminating infection with the pathogen of interest.
- The ability to maintain facility biosecurity and eliminate exposure to amphibians that are not SPF.

- The use of techniques that separate developing or juvenile animals from infected parents or other sources of contamination before they can become infected with the pathogen of interest. None of these techniques have been validated for use in amphibians or specifically for amphibian pathogens. Techniques include:
 - Removal and/or disinfection of eggs following removal from a contaminated environment.
www.oie.int/eng/normes/fmanual/1.1.3_DISINFECTION.pdf
 - Removal of larvae by caesarian section (for viviparous amphibian species such as the Kihansi Spray Toad, *Nectophrynoides asperginis*).

Approaches that might be used to create SPF populations of amphibians for the amphibian chytrid fungus or ranaviruses are presented below.

Creating Captive Populations Free of the Amphibian Chytrid Fungus

Creation of captive amphibian populations free of infection with the amphibian chytrid fungus is important for:

- Success and sustainability of amphibian survival assurance populations, especially for those species that are very sensitive to lethal chytrid fungal infections and are threatened with extinction because of chytridiomycosis.
- Providing a source of animals free of the amphibian chytrid fungus for use in the pet trade, human consumption and as laboratory animals. This minimizes the potential for captive animals to act as a source of infection for new amphibian populations.

A strategy for Creating Captive Populations Free of the Amphibian Chytrid Fungus

1. The presence or absence of the amphibian chytrid fungus must be determined for the captive population. Testing of animals using the polymerase chain reaction (PCR) will be necessary (see Section 7.3) unless the population is already known to be infected as indicated by the results of ongoing necropsy surveillance or prior PCR testing.

- It is important that PCR testing of a population be designed to collect an appropriate sample size to be confident in the absence of infection.
- Multiple PCR tests are required to determine if individual animals are definitively free of infection. A single negative test result is insufficient for purposes of creating chytrid fungus free populations. False-negative test results occur in animals that have low-level or subclinical infections (see Section 7.3). Individual animals (small populations) or appropriate sample sizes of large populations should initially be tested at least twice and as many as 3 times over a 2-week period. Use of protocols that pool swab samples from multiple animals housed in the same enclosure may be helpful for reducing the costs of testing.

- Animals that die should be submitted for necropsy examination and histopathology to determine if death was due to chytridiomycosis (see Chapter 9).
2. If animals are all PCR-negative in the testing performed in step 1 and necropsy findings in animals that die are negative for chytridiomycosis, a preliminary determination of chytrid fungus-free status can be made. **Go to step # 5.**
 3. If PCR positive animals are identified or if deaths due to chytridiomycosis are found on necropsy examination, the entire population is considered to be infected with the chytrid fungus. This determination is regardless of the test results for any individual animal or animals. In other words, animals that test negative by PCR in this situation are still considered to be infected with the chytrid fungus if other animals in the group test positive. The entire population of animals should be treated with antifungal medication using the protocols discussed as discussed above in Section 8.1.
 4. After treatment of the population with antifungal medication is completed wait for a minimum of two weeks before repeating PCR testing as described in step # 1. If the post-treatment testing series detects PCR-positive animals, step # 3 is repeated for the entire population until PCR tests for the population are negative. If the post-treatment testing series is negative for the entire population a preliminary determination of chytrid fungus-free status can be made.
 5. Populations with a preliminary determination of chytrid fungus free status as described in step # 1 or step # 4 should be monitored by a disease surveillance program for an extended period (e.g., 6 months to 1 year) of time before final determination of Bd-free status. Disease surveillance can include necropsy and histopathology as well as sporadic PCR testing of animals in the population. If PCR-positive animals are identified or if chytridiomycosis is discovered on necropsy examination, step # 3 is repeated.
 6. It is important that PCR negative amphibian populations be kept in permanent isolation from animals infected with the chytrid fungus or for which the infection status is unknown. Biosecurity practices outlined in Chapter 4 may be helpful. Before any new animals are added to the population it is important that they are subjected to a thorough quarantine process (see Chapter 6) which includes PCR testing for the amphibian chytrid fungus.

Creating Captive Populations Free of Ranaviruses

The creation of *Ranavirus*-free amphibian populations is suggested by the World Organization for Animal Health (OIE) for control of ranaviral infections in farmed amphibians. The processes needed to create Ranavirus SPF populations is less clear when compared to the amphibian chytrid fungus because of the lack of a validated diagnostic test for use in living animals.

- *Ranavirus* SPF populations will also have application to animals raised in large numbers for the pet trade.
- For survival assurance populations and amphibian reintroduction programs, *Ranavirus* SPF populations will likely only be necessary in situations where the captive population has acquired a *Ranavirus* that does not normally circulate in the wild population or is infected with a *Ranavirus* that consistently causes significant mortality in the captive population.

Strategies for Creating Captive Populations Free of Ranaviruses

1. Determine the presence or absence of ranaviral infections in the captive population.

Suggested methods include:

- Surveillance by necropsy and histopathology for deaths in the population that have signs that could be suggestive of *Ranavirus* infection. These include: hemorrhages in multiple tissues; degeneration and necrosis in liver, kidney, gastrointestinal tract or hematopoietic tissue; and proliferative or ulcerative skin conditions.
- Perform PCR testing on tissues (e.g., liver and kidney) collected at necropsy from all animals that die and/or from animals that are culled from the population for disease surveillance purposes. See Section 7.4)
- Collection of samples for PCR testing from outwardly healthy living animals has not been validated and is not recommended as the sole diagnostic test for determining the *Ranavirus* infection status of a population.
- Surveillance should be performed over an extended period (e.g., 1 year).
- Groups of animals to be used for creation of an SPF population should be held in long-term isolation from other amphibians (see Section 4.9)

2. If surveillance for ranaviruses described in step # 1 is negative, the population may be tentatively considered to be *Ranavirus*-free. **Go to step # 4.**

3. If surveillance for ranaviruses described in step # 1 is positive for evidence of *Ranavirus* infection in the population, the following measures may be helpful:

- If sick animals are present separate these individuals from clinically healthy animals.
- Increase surveillance efforts as described in step # 1. Elective culling of animals for disease surveillance (PCR testing of tissues) should be considered.
- If individual groups of animals in the population test negative for evidence of *Ranavirus* infection, they should be separated from groups that test positive. Good biosecurity practices that isolate these negative groups from positive groups should be instituted (see Section 4.9).

- Surveillance measures as described in step # 1 are continued for groups that test negative. Surveillance should be for an extended period of time (e.g., 6 months to 1 year). If these groups remain PCR negative they may be tentatively considered to be *Ranavirus*-free. At this stage, **go to step # 4**.
 - Consider re-deriving captive populations by removal of eggs from contaminated environments or populations. A potential caveat is if *Ranavirus* infections can have vertical transmission (unknown).
4. It is important that *Ranavirus* SPF amphibian populations be kept in permanent isolation from animals infected or potentially infected with ranaviruses. Before any new animals are added to the population it is important that they are subjected to a thorough quarantine process.

8.4 PARASITE MONITORING AND TREATMENT

Although it is not always desirable to completely eliminate natural parasite loads in animals destined for reintroduction to the wild (Lyles and Dobson, 1993; Cunningham, 1996), control programs for some endoparasites are necessary under captive conditions. Of particular importance are infections with rhabditiform nematodes such as the amphibian lungworm *Rhabdias*, and the intestinal nematode *Strongyloides*. These are common subclinical infections in wild amphibians, but cause serious problems in groups of animals recently brought into captivity from the wild (Lee et al., 2006; Pessier, 2008).

- Many amphibians have adapted to survive with low levels of nematode parasite burdens in the wild. In turn, parasites produce very large numbers of offspring to increase likelihood that one offspring will eventually encounter a viable host. When an infected animal is brought in to captivity, the animal is repeatedly exposed to heavy environmental contamination with high numbers of infective parasite ova or larvae.
- In the case of rhabditiform nematodes, these parasites have a direct life cycle which is completed in as little as 48 hrs, and hyperinfections can easily occur. (Poynton and Whitaker, 2001).
- Rhabditiform nematodes are controlled in captive situations by combinations of fecal parasite monitoring, good enclosure and facility hygiene and anthelmintic treatment.

Parasite Monitoring

Monitoring of fecal samples for evidence of internal parasitism is an important part of a parasite control program (see Section 7.7)

- During quarantine for new animals the goal is to reduce the parasite burden as much as possible to avoid introducing hyperinfected animals to the established captive populations. Fecal monitoring for quarantine is discussed in detail in Section 6. 11.

- Animals in captive populations that have successfully passed through quarantine are monitored by fecal examination at least every 6–12 months, or more frequently as needed to achieve a desired level of parasite control.
 - In addition to routine checks, all animals that present with weight loss, loss of appetite, ill thrift, etc, should have a fecal sample checked.
 - Fecal examinations are also performed more frequently if necropsy surveillance of the population (see Chapter) indicates that parasite problems are an important cause of illness or death.
- For the rhabditiform nematodes, a standardized scoring system is employed to accurately track parasite levels. One fecal scoring system for rhabditiform nematode larvae is proposed below. This is admittedly a subjective system and other approaches could be used. Use of a consistent system within a facility is most important.

This system for rhabditiform nematode larvae is used with direct wet-mount examination of feces:

- 1+ = 1 or fewer larva identified for every 3 or more low powered fields (10x objective)
- 2+ = 1 larva identified for every 2–3 low powered fields
- 3+ = 1 larva identified for every 1–2 low powered fields
- 4+ = 1 or more larvae identified in every low powered field; for 4+ samples, try to record an approximate number of how many larvae are seen per low powered field

Note: as the slide is scanned under low power (10x objective), each new position on the slide is considered one low-powered field. A score is assigned based on an average of all fields.

It is recommended that all animals in an established collection with 3+ or higher result should be treated. All animals receiving parasite treatment should have a recheck fecal exam 1 week after the last treatment. If a fecal sample is positive in an enclosure containing more than one animal, consider all animals within that enclosure to be positive.

Parasite Treatment

Treatment of the rhabditiform nematodes requires attention to hygiene of the animal enclosure in order to control the free-living environmental stages of the parasite as well as administration of anthelmintic medications to reduce internal parasite loads.

- For very heavily infected animals consider housing within temporary enclosures that are easily cleaned and disinfected and contain minimal amounts of organic substrates. Feces should be removed from the enclosure daily and substrates or

entire enclosures should be replaced and cleaned approximately every 2 days during treatment.

- For long-term parasite control, animal enclosures should be designed for easy cleaning and removal of fecal waste.
 - Use of bottom drilled enclosures with false bottoms allows for frequent flushing (e.g., weekly) of enclosure substrates.
 - Fecal material should be manually removed from surfaces of plants or other enclosure substrates.
- For administration of anthelmintic medications, obtain an accurate body weight of each animal in grams.
- Prior to use of a medication in a new species, it is prudent to treat only a small number of animals prior to treating the entire collection. All of the medications listed below have proven safe in a wide range of species, though it is possible for individual species sensitivity and toxicity to occur. Further, any of the medications may be toxic if overdosed; careful attention to accurate body weights, calculations of doses, and preparation of dilutions is essential.
- Medication options include:
 - **Drontal Plus**[®] Bayer HealthCare Animal Health Division. A combination product containing praziquantel, pyrantel pamoate and febantel. A suspension is compounded using commercially available oral tablets for dogs. The suspension is formulated to provide 2.25 mg/ml of the pyrantel component of the product. Dosage is 0.01 ml (or 10 microliters) orally, per 1 g of body weight. Repeat dosage in 2–3 weeks. This protocol has been used safely and effectively in Panamanian anurans brought into survival assurance populations (Gagliardo et al., 2008). Use of a micropipette is useful in very small animals. A soft guitar pick, or similar thin, blunted semi-rigid plastic device is used to gently open the animals' mouth for direct oral dosing.
 - **Fenbendazole** (Panacur[®]) oral suspension 10% (100 mg/ml). A commonly used anthelmintic in veterinary medicine with a wide safety profile. Dosage is 50–100 mg/kg. 0.5–1.0 microliter of 100mg/ml suspension per 1 g of body weight (50–100 mg/kg), administered orally (as described for Drontal Plus). Repeat in 10–14 days. Alternatively, for very small animals, use fenbendazole 22% granules (222mg/g) to dust prey items. Offer dusted prey items once a day for five days and repeat in 2–3 weeks.
 - **Levamisole**. This is a commonly used anthelmintic for domestic livestock and is available in many countries as an injectable solution. Dosage is 10mg/kg (equivalent to 0.01mg levamisole per g of body weight), applied topically to the skin. Application to the skin has advantages for animals that are easily stressed and do not tolerate oral administration of medication. A treatment solution is made by diluting the commercially available injectable solution to 10 mg per milliliter. Using this solution, apply 0.01ml per 10 g body weight. For animals

weighing less than 10 grams, use a micropipette to deliver 1 microliter per 1 gram of body weight. After application of solution on skin, rinse skin with fresh water after 1 hour of contact. Monitor animal for signs of paralysis – if this occurs, rinse animal thoroughly and maintain in a well oxygenated, cool environment until recovery. Repeat treatment every 14 days for a total of 2–3 doses. This medication has been used safely on a wide variety of captive anurans and caudates in zoological collections and *ex situ* assurance colonies. Effectiveness has varied and re-check of fecal samples 1–2 weeks after completion of treatment is necessary to verify effect, as with any antiparasitic treatment.

- **Ivermectin.** A 1% (10 mg/ml) ivermectin injectable solution is available (Ivomec[®]). This preparation may be given topically, injectably, or orally. Dosage for most amphibians is 0.2 mg/kg, equivalent to 0.02 microliters of the 1% ivermectin injectable per gram of body weight. Administration will require dilution of the 1% product in order to accurately measure the dose. Alternatively, an ivermectin bath may be prepared to a concentration of 10mg/L (1ml of 1% ivermectin injectable in 1 liter of water). Animals may be placed in the bath for 60 minutes, repeated in 7 days. There have been anecdotal reports of toxicity to ivermectin in some species, so care should be taken when applying this solution to unfamiliar species.

8.5 ELECTROLYTE FORMULAS

Amphibians with chytridiomycosis or other pathogens can have significant electrolyte imbalances. Therefore, in addition to antifungal treatment, affected animals benefit from fluid and electrolyte therapy. Approaches to amphibian fluid therapy have been reviewed elsewhere (Wright & Whitaker, 2001). For mildly to moderately affected animals, electrolyte baths such as amphibian Ringer's solution applied continuously for aquatic species or supplied as a water source for terrestrial species, may be adequate. For depressed or moribund animals, intracoelomic administration of balanced (non-lactated) electrolyte solutions diluted 1:1 or 2:1 with 5% dextrose may be necessary.

- **Recipe for Amphibian Ringer's solution (1 liter)**
 - Sodium chloride (NaCl), 6.6 grams
 - Potassium chloride (KCl), 0.15 grams
 - Calcium chloride (CaCl₂), 0.15 grams
 - Sodium bicarbonate (NaHCO₃), 0.2 grams

Add distilled water to make 1 liter of solution. Mix solution thoroughly to ensure that all crystals are dissolved. Agitate thoroughly before use. Keep in a closed container to reduce evaporation (Wright and Whitaker 2001). If dry chemicals are purchased, it is convenient to premix the appropriate chemicals in separate individual bags that are ready to be added to water. There are also convenient and inexpensive premixed

concentrated liquid stock solutions commercially available:
[http://www.enasco.com/product/SA09708\(LM\)M](http://www.enasco.com/product/SA09708(LM)M)

- **Recipe for Whitaker-Wright Solution (1 liter of 100% stock solution)**

- Sodium chloride (NaCl), 113 grams
- Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 8.6 grams
- Calcium chloride (CaCl_2), 4.2 grams
- Potassium chloride (KCl), 1.7 grams

“Dissolve crystals thoroughly in distilled water. Keep container covered to prevent evaporation. Add Trizma (7.4) base, fish grade, as needed to stabilize pH of solution between 7.0 and 7.3” (Wright and Whitaker, 2001). This is a stock solution that must be diluted before use. A 12% Whitaker-Wright solution has been used as an oral electrolyte supplement for frogs with chytridiomycosis (Voyles et al., 2009; Berger et al., 2010). A 12% solution is made by adding 12 ml of stock solution to 88 ml of distilled water.

8.6 EUTHANASIA

A variety of euthanasia methods have been described for amphibians including bath immersion in tricaine methanesulfonate (MS-222) or benzocaine; injection of barbituates such as pentobarbital; and physical methods such as pithing. These methods have been discussed in reviews of animal euthanasia methods (www.avma.org/issues/animal_welfare/euthanasia.pdf)

Immersion in tricaine methanesulfonate (MS-222) (Finquel[®], Argent Chemical Laboratories) is one of the least stressful euthanasia methods and interferes the least with diagnostic laboratory testing such as necropsy and histopathology. Animals are placed into a bath containing 1.0 to 3.0 grams of MS-222 per liter. Animals are left in solution until they are unresponsive to stimulation and there is evidence that respiration and cardiac activity have ceased. Benzocaine hydrochloride is a related drug to MS-222 and can be administered by bath at least 250mg per liter.

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Chapter 9

AMPHIBIAN NECROPSY

9.0 INTRODUCTION

Necropsy (postmortem examination) is a major component of disease surveillance programs for amphibian survival assurance colonies and an important tool for disease risk assessments.

- Ideally, a complete necropsy should be performed on most animals that die in amphibian survival assurance colonies.
- Necropsies are also performed on animals culled from populations in order to collect information for disease surveillance.
- A complete necropsy includes histological examination (histopathology) of all major organs and the skin.

Benefits of necropsy and histopathology for amphibian survival assurance colonies include the following:

- Histopathology can detect a wide range of both infectious and non-infectious diseases (including evidence of nutritional deficiencies common in survival assurance colonies). It is the diagnostic method that is most likely to detect new or unexpected disease problems for which specific tests (e.g., PCR for amphibian chytrid fungus) are not available.
- In addition to being a tool for disease risk-assessment, data collected from necropsy is used by veterinarians and facility managers to determine the most significant health problems affecting a survival assurance colony. This allows focus of management efforts towards those actions (e.g., diet correction; treatment of a specific infection) that will be of most benefit for maintaining healthy and self-sustaining survival assurance colonies.

Not all facilities will have immediate access to a Veterinary Pathologist or even an on-site veterinarian that can perform necropsy examinations.

- In these situations it is still important to collect postmortem samples that can be preserved and used at a later date when veterinary or pathology expertise becomes available
- The **Carcass Fixation** necropsy method described in the **Performing the Necropsy** section can easily be taught to lay staff without a biology or veterinary background.

- Necropsy samples are an irreplaceable resource for understanding disease problems in survival assurance colonies. Therefore, it is much better to always collect and preserve samples than to later regret not collecting the samples at all.
- This section reviews amphibian necropsy procedures with an emphasis on sample collection for survival assurance colonies that do not have on-site veterinary support. Detailed reviews of amphibian and reptile necropsy are available (Nichols, 2001; Pessier and Pinkerton, 2003; Terrell and Stacy, 2007). General guidelines for collection of necropsy samples from wild animals are available and contain useful information (www.wcs.org/~media/Files/pdfs/necropsy2.ashx).
- Special considerations for mortality events in which multiple animals are found dying or dead will also be discussed.

9.1 NECROPSY PREPARATION

A necropsy should be performed as soon as possible after death of the animal. Decomposition (autolysis) of the tissues occurs rapidly in amphibians and the sooner that samples can be preserved, the more likely that histopathology and other diagnostic testing will provide useful information.

- If necropsy is not performed immediately, the carcass should be stored under refrigeration (2–8 °C) for up to 24–72 hours.
- Carcasses should not be frozen unless there is no other alternative or unless they are required for other diagnostic testing (see Investigation of Mortality Events below). Freezing can create significant artifacts that interfere with interpretation of histopathology.
- Carcasses stored in the refrigerator should be placed into a leak-proof plastic bag or other sealed container. Containers are labeled with the species name, individual animal identification number and date.
- Necropsies should be performed in a clean location away from areas involved in the housing of animals, food preparation or insect culture, or processing and handling of animal enclosures and substrates.

9.2 MATERIALS NEEDED

- **Tissue fixative.** A solution of neutral buffered formalin is best for preservation of tissues for histopathology. The formula to make 1 liter of neutral buffered formalin is:
 - 100 ml 37% or 40% formaldehyde.
 - 900 ml distilled water.
 - 4.0 g monobasic sodium phosphate.
 - 6.5 g dibasic sodium phosphate.

Neutral buffered formalin can also be obtained pre-mixed from chemical or medical suppliers. If formalin is not available, a solution of 70–90% ethanol can be substituted as a tissue fixative.

- **Supplies for collection and storage of frozen tissue samples.** Examples include:
 - Whirl-Pak® style bags (Nasco, USA, www.enasco.com). Useful for freezing larger volume samples or entire carcasses.
 - Cryovials such as Nunc CryoTubes™ or Vangard Cryos™ (Sumitomo Bakelite Co., Ltd. Japan, www.sumibe.co.jp/english/). Useful for freezing individual organ or tissue samples.
- **Instruments.** For most amphibians only a few basic instruments are required to perform a complete necropsy and include:
 - a scalpel handle and scalpel blades.
 - scissors (multiple pairs are suggested; dull pairs of scissors can be used for cutting bone).
 - tissue forceps (“tweezers”).
 - Centimeter ruler (for obtaining measurements of the carcass and any lesions).
 - Cutting board (for use as a work surface). Plastic cutting boards are better because they are more easily cleaned and disinfected.
 - Digital scale (helpful to obtain body weights). The scale should not be the same scale used to weigh living animals.
- **Protective Equipment.** These protect the person doing the necropsy from exposure to important pathogens and help to ensure that infectious materials are not moved from the necropsy area to areas that house living animals. Suggested equipment includes:
 - Disposable latex or nitrile gloves.
 - Laboratory coat or plastic apron to protect clothing.
- **Alcohol lamp or butane burner.** For sterilizing instruments used in collecting samples for microbiology or molecular diagnostic tests.
- **Standardized necropsy form.** To record specimen information and necropsy observations.

9.3 PERFORMING THE NECROPSY

1. Prior to necropsy obtain a body weight and a snout-vent measurement. An external examination should be performed with special attention given to:

- **The general condition of the carcass.** Things to consider include degree of postmortem decomposition; discharges from the nose, mouth or cloaca; and if bones are prominent (suggesting emaciation).

- **Condition of the skin surfaces** (e.g., presence of discoloration; excessively shedding skin; increased mucus; skin nodules; areas of ulceration; external parasites).

2. If skin lesions are present it may be beneficial to perform a skin scraping for wet mount and cytology (see Section 7.3).

- In addition to the amphibian chytrid fungus, skin scrapings can help to diagnose a variety of other bacterial, fungal and parasitic infections (Pessier, 2007).
- Areas of the skin used for a skin scraping should be adjacent, but not identical, to the areas sampled for histopathology.
- The increased availability of affordable digital camera systems for microscopes may enable survival assurance colonies that do not have on-site veterinary support to photograph selected areas from a skin scraping and email those images to an off-site consulting veterinarian or pathologist for diagnosis.

3. Next, using a clean cutting board or other easily cleaned and disinfected work surface, place the animal in dorsal recumbancy (on its back).

- Using a new sterile or freshly cleaned and disinfected scalpel or scissors make an incision on the ventral midline from the jaw to the level of the cloaca.
- Reflect the skin on either side of the incision to reveal the abdominal musculature.
- Carefully incise the abdominal musculature (body wall) along the midline to enter the coelomic cavity, but do not touch the underlying viscera (Figure 9.1).

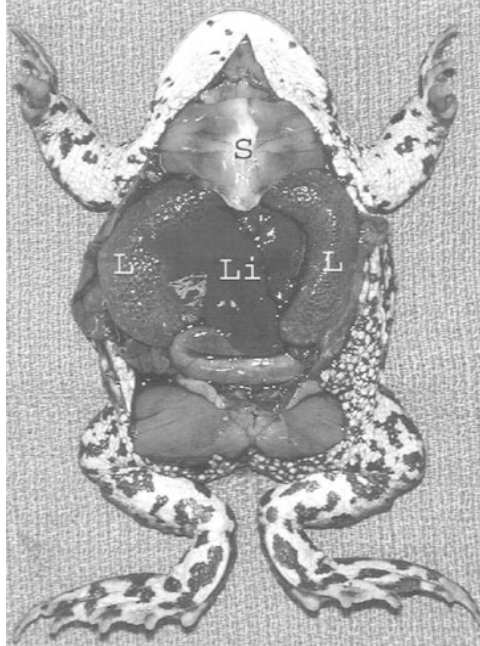


Figure 9.1: Necropsy of a toad (*Anaxyrus boreas*). Showing a ventral midline incision from the jaw to the cloaca and into the coelomic cavity. Labeled are the lungs (L), sternum (S) which overlies the heart, and liver (Li). Image from Pessier and Pinkerton (2003).

4. If fluid is observed to be present in either the subcutaneous spaces (lymph sacs in anurans) or within the coelomic cavity, collect a sample using a sterile syringe and needle.
 - If the services of an outside clinical pathology laboratory are available, it may be useful to submit the fluid for bacterial culture and/or for fluid analysis (cell count, total protein and cytology; Pessier, 2007).
5. Following incision into the coelomic cavity and exposure of the visceral organs, use a dull pair of scissors to cut and remove the sternum. This exposes the heart and the cranial most aspect of the lungs.
6. After exposure of the visceral organs and heart, sterile samples can be collected for microbiology, molecular diagnostic testing (e.g., PCR for *Ranavirus*) or to be stored frozen if desired.
 - If the capability exists to save frozen tissue samples (freezer or liquid nitrogen) the routine collection of a small portion of the liver is recommended (for location of the liver see Figure 9.1). An ultra-cold freezer (-70°C or below) is preferable, but are expensive and may not be available everywhere. This sample is placed into a cryovial or Whirl-Pak bag labeled with the species name, animal ID number and date. This sample can be saved and used for diagnostic testing at a later date if required.

- If obvious lesions are identified at necropsy such as enlarged organs, visceral nodules, or areas of visceral discoloration, collect part of the lesion for freezing and the remainder for histopathology as described below. If the lesion is very small (less than 5mm) it is better to save it for histopathology than it is to save a frozen sample.
- Under some circumstances it is desirable to save multiple different frozen tissue samples (see Section 9.5 below).
- If it is desired to submit samples for bacteriology. Good samples to submit include heart blood or liver.
- After the carcass is opened, a decision can be made to perform either the Carcass Fixation or Dissection necropsy technique.

Carcass Fixation Necropsy Method

This is the preferred technique for facilities that do not have an on-site veterinarian (or other individual with knowledge of amphibian anatomy) or for most small amphibians (< 20 g body weight).

- This method has been used successfully for animals larger than 20 grams, but there is a greater risk of poor tissue preservation if the coelomic cavity is not opened sufficiently or if too little fixative solution is used for preservation.
- **To perform the Carcass Fixation method:**
 - Open the coelomic cavity and expose the visceral organs as described above.
 - Obtain tissues for freezing or microbiologic analysis as described above.
 - Immerse the opened carcass into fixative solution. For optimum tissue preservation, use a ratio of approximately 1 part carcass to 9 parts tissue fixative.
 - Keep the carcass in fixative solution until shipment to a Veterinary Pathologist for histopathology.

If the carcass is large (> 20–30 g body weight) the procedure is modified to allow better tissue preservation by:

- Removing the head from the carcass using a scalpel or dull pair of scissors. This allows fixative solution to better penetrate into the brain.
- Using a syringe and needle to inject small amounts fixative solution into the stomach and intestinal tract.

Both of these modifications require a degree of comfort with dissection techniques or knowledge of amphibian anatomy and can be considered optional.

Dissection Necropsy Method

This method is desirable for larger animals (> 20 g body weight) and for situations where a veterinarian or other individual with a good working knowledge of amphibian anatomy is available to perform the necropsy. The procedure is a thorough dissection of the carcass and preservation of representative pieces from different organs. If the dissection method is performed on very small animals, the use of a dissecting microscope is helpful for appreciating subtle details.

- **To perform the Dissection method:**
 - Open the coelomic cavity and expose the visceral organs as described above.
 - Obtain tissues for freezing or microbiologic analysis as described above.
 - Samples from all major organ systems should be obtained and placed into fixative solution. The ratio of tissue to fixative should be one part tissue to 9 parts fixative. Ideally, pieces of tissue should not exceed 0.5 cm in thickness. Larger organs can be serially sliced and examined for lesions. A list of the tissues that should be routinely collected into fixative for histopathology is found below.
 - Notes on necropsy findings should be recorded on a standardized necropsy form

Table 9.1: Tissues to be collected for amphibian histopathology

Skin (multiple sections to include dorsal and ventral body as well as any lesions)	Gallbladder
Lung or Gill	Skeletal Muscle
Heart	Bone
Liver	Peripheral Nerve
Spleen	Urinary Bladder
	Gonad (testis or ovary)
Kidney	Cloaca
Stomach	Eye
Small and Large Intestine	Larynx
Pancreas	
Brain	

9.4 ANATOMICAL NOTES FOR AMPHIBIAN NECROPSIES

A few unique features of amphibian anatomy and physiology are valuable to consider or remember when performing a necropsy examination. These include:

- Unlike other vertebrate animals the skin is important for a variety of physiologic processes such as water and electrolyte absorption, osmoregulation and for some species, respiration. Therefore, it is extremely important to evaluate the skin (especially by histopathology) whenever an amphibian necropsy is performed.
- Body fat stores are predominantly held in the coelomic (gonadal) fat bodies located cranial to the gonads. In anurans, these fat bodies appear as strands or “fingers” of white to yellow tissue (Figure 9.3).
 - Most healthy animals should have some identifiable coelomic fat bodies. If fat bodies are small or cannot be identified then the animal is usually considered to be in poor overall nutritional condition. Causes of poor nutritional condition include poor food intake due to maladaptation to captivity, stress or excessive competition for food or underlying disease conditions.
- Most amphibians have paired lungs, however, the plethodontid salamanders are lungless and some caecilians have a functioning right lung and only a vestigial left lung.
- In animals from the family Bufonidae, males have a small aggregate of ovarian tissue at the cranial pole of the testis (Bidder’s organ; Figure 9.2).
- The ovaries of anurans can be very large and fill the coelomic cavity. In some species the ovaries are pigmented black (Figure 9.3). The oviducts are prominent and appear as coils of tissue around the ovary (Figure 9.3).
- Because the urinary bladder is very thin-walled and translucent it is often overlooked on necropsy examination. Samples of the urinary bladder can be obtained for histopathology by removing soft tissues within the pelvic canal.

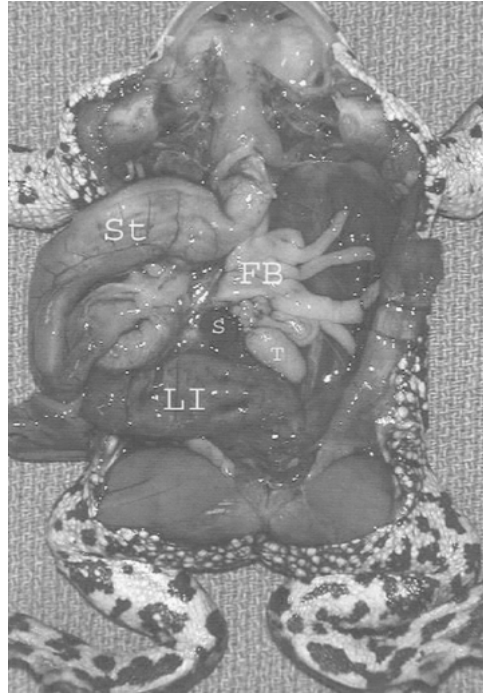


Figure 9.2: Necropsy of a toad (*Anaxyrus boreas*). The liver has been removed and the gastrointestinal tract has been moved to show the coelomic fat bodies (FB). Also labeled are the stomach (St), large intestine (LI), spleen (S), and testis (T). Bidder's Organ slightly visible at the cranial pole of the testis adjacent to the spleen. Image from Pessier and Pinkerton (2003).

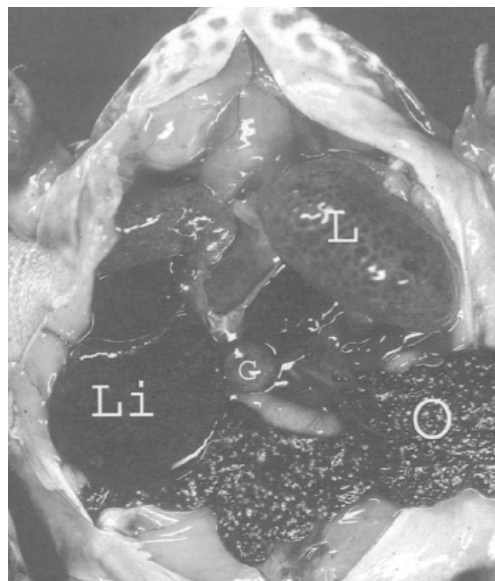


Figure 9.4: Necropsy of an African bullfrog (*Pyxicephalus adspersus*) showing the black pigmented ovaries that partially fill the coelomic cavity (O). Also shown are lung (L), liver (Li) and gallbladder (G). Also labeled are the stomach (St), large intestine (LI), spleen (S) and testis (T). Image from Pessier and Pinkerton (2003).

9.5 SAMPLE COLLECTION DURING MORTALITY EVENTS

Mortality events where multiple animals are found dying or dead are observed in amphibian survival assurance colonies as well as wild amphibian populations. Although well-known infectious diseases of amphibians (e.g., chytridiomycosis or *Ranavirus* infection) may be strongly suspected, it is important to keep an open mind and always consider other potential causes. Many different disease conditions can initially look very similar and require laboratory investigation to achieve a definitive diagnosis.

- The initial goal of investigating mortality events is to collect and preserve representative samples that can be used for the different types of laboratory techniques that may be needed.

Complex protocols can be designed for sample collection during mortality events—especially if veterinary guidance is available—however, a simple and basic approach that will be suitable for most situations is to:

- Preserve one set of tissues (or carcasses) in a fixative solution for histopathology
- Preserve a second set of tissues (or carcasses) frozen that can be used for molecular diagnostic techniques (such as PCR), to isolate infectious agents in culture or for toxicological analyses.

Some guidelines for sample collection during mortality events:

- If veterinary guidance is not available or if animals are small, perform the Carcass Fixation necropsy method (described above) on one-half to two-thirds of the dead animals. For the remaining animals, freeze the entire carcasses as soon as possible and label with the species name, individual identification number and date.
- If veterinary guidance or an individual experienced with amphibian anatomy is available, perform the Dissection necropsy method (described above) on the dead animals.
 - In addition to saving samples from all major organs in fixative solution for histopathology save additional samples of individual organs frozen.
 - Suggested samples for freezing include liver, kidney, lung, intestine, brain and any tissue thought to be abnormal during dissection (e.g., enlarged or discolored organs or organ nodules). In addition, stomach contents, coelomic fat bodies and skeletal muscle can also be saved, especially if exposure to a toxic substance is a possibility.
 - Organ samples are saved in Whirl-Pak® style bags (Nasco, USA, www.enasco.com) or cryovials such as Nunc CryoTubes™ or Vanguard Cryos™ (Sumitomo Bakelite Co., Ltd. Japan, www.sumibe.co.jp/english/). Containers should be labeled with the species name, individual animal ID number, specimen type, and date.

- For freezing of entire carcasses or individual tissue samples, ultracold temperatures (-70°C or below) or in liquid nitrogen are preferable, however, regular household freezer temperatures are sufficient for short-term storage. As a last resort, if a freezer or liquid nitrogen is unavailable, fixation of carcasses or tissue samples in 70% ethanol (instead of formalin) may still allow application of some molecular diagnostic techniques.
- If dying animals are found, consideration should be given to euthanasia of some of these individuals for necropsy and sample collection (see Section 8.6). This provides very fresh samples that are ideal for most laboratory methods used for disease investigation.

BASIC SAMPLE COLLECTION PROTOCOL FOR AMPHIBIAN MORTALITY EVENTS (Veterinarian Not Available, or Field Situation with Limited Equipment)

- For half of the dead animals, make an incision into the coelomic cavity and expose the internal organs. For very small animals or if a knife is not available, just fix the carcasses intact. Place the opened carcass into a fixative solution such as 10% neutral buffered formalin (preferred) or 70% ethanol. The ideal ratio is one part animal carcass to 9 parts fixative solution.
- For the other half of the dead animals, freeze the entire carcasses or keep them cool (such as a portable ice-chest) until they can be transported to a location where freezing is possible.
- It is always better to save both fixed (formalin or ethanol) and frozen samples. If this is not possible, preference should be given to saving tissues fixed in formalin or ethanol. Saving only frozen samples should be a last resort (but is better than no samples at all).
- If freezing of samples is not possible, fixation in ethanol may allow for both histopathology as well as some molecular diagnostic tests (e.g., PCR)

9.6 SHIPMENT OF SAMPLES

For shipment of tissues that have been preserved in a fixative solution. Once carcasses or tissues have been in formalin or other fixative solution for a minimum of 48 hours, they are removed from fixative, wrapped in paper towels or gauze moistened with fixative, packed into sealed plastic bags and shipped to a pathologist. This minimizes the potential for leakage during shipment and reduces package weight (and shipment costs).

- Materials should be shipped in a manner that follows International Air Transport Association (IATA) regulations for Dangerous/Hazardous Materials. Some general guidelines include:
 - Samples should be enclosed in a primary receptacle that is leak-proof.
 - The primary receptacle is then placed within a leak-proof secondary receptacle.

- An absorbent material (e.g., paper towels) should be placed between the primary and secondary receptacles. The volume of material should be sufficient to absorb all of the fluid within the primary receptacle.
- Major shipping companies have guidelines available to help with proper shipping of biological samples. More information available here: <http://images.fedex.com/downloads/shared/packagingtips/pointers>

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Appendix 1

METHODS TO INDIVIDUALLY IDENTIFY THE AMPHIBIAN SPECIMEN

R. Andrew Odum and Edythe Sonntag

INTRODUCTION

The quality of management of animals is greatly enhanced when specimens can be identified as individuals. Without this ability to consistently identify each specimen it becomes impossible to maintain medical histories, pedigree data, and other pertinent information that is directly related to the specimen. Inability to identify individuals will also impact gene diversity maintained in a captive population (see section on Genetic Management).

For the past twenty-five years zoo populations of endangered species have been managed to maintain genetic diversity through selective breedings. Genetic population management is most efficient at the individual level with full pedigrees (Schad, 2007). This requires that each individual can be identified through its life and that parentage can be established for all offspring.

Maintaining the long term identify of an animal requires some type of recordable identifier to connect the individual with its records. In zoos, this is usually an accession number and in some rare cases for particularly noteworthy specimens of the Amphibia, a name. These identifiers can be used as a key for information stored in the records system and thus establish its pedigree.

Individual identification techniques can be divided into two general categories: Those that are invasive and those that are not. It is important to note that all techniques are not entirely full proof. All have failed under some conditions, and in invasive techniques, mortalities have occurred. Some invasive techniques can cause permanent deficits, impair physical activity, and decrease survivability (i.e., tissue amputation). These techniques need to be individually evaluated for the circumstances for their proposed use prior to implementation.

NON-INVASIVE TECHNIQUES

Animal Color and Pattern

One of the simplest and most effective methods for identify amphibians is by their pattern and coloration. In many species, once the animal reaches its adult form, its pattern, marking, glandular structures and coloration usually stabilize for the remainder of their life. Although there might be some ontogenetic changes as the animal ages (i.e., darkening), its earlier markings are usually still visible. These patterns

can be documented either by drawing (if minimal talent is available) or by photographing the animal. Depending on the species, it may be best to use the dorsum, venter, or lateral areas to delineate specimen differences. In the case of animals with “warty” or granular skin, the position and numbers of these features, as well as the coloration are excellent unique identifiers for animals.

The patterns of juvenile animals may change as the animal matures; however, with repeated photographs being taken every 1-4 months, the staff at the Detroit Zoo successfully tracked the identification of a group of juvenile emperor newts through adulthood and saw minimal changes. With regular updating of the records, it is possible to use photographic pattern IDs on juvenile animals in species where there is ontogenetic changes in pattern and color. However, reliance on this method must include a commitment to regularly updating images during growth.

There are limitations for this technique. Obviously if the animals are visually indistinguishable, this technique is ineffectual. In addition as the number of animals in an enclosure increases, it becomes more tedious to determine one animal from the group, particularly if the differences between individual specimens are minimal. Another important factor is size. If the specimens are very small, it is difficult to identify differences without a magnification device, adding the need for instrumentation and specimen restraint. Depending on the species, this technique becomes somewhat ineffective when there are more than five to ten specimens housed together.

There are anecdotal accounts of using xerographic copy machines of fossorial caecilians to document annular ring patterns. Though this method of identification generally disturbed the office staff and soiled the copier, it was effective and less stressful than trying to hold the animals for photographing or drawing. The animal was placed on the clean glass of the copier and a moist towel was placed over it for restraint.

As technology has improved and digital cameras have become the norm, pattern recognition has moved well beyond researchers with hand drawn renditions of the animals they study. Gamble et al. (2008) have developed a pattern recognition algorithm which uses photographs of marbled salamanders (*Ambystoma opacum*) taken in the field. Tests of the system proved successful for the identification of an individual in only about a minute with 95% accuracy in a database of 1000 images.

Isolation

Another simple technique for maintaining individual identification is by separating animals into different enclosures. If there is only one animal in the cage, you can easily know who it is. This can also be applied to sexual pairs housed together in the same enclosures, as long as you can determine the sex of the individual specimens (which may or may not be the case in some species of amphibians). By attaching a card with the accession number on the cage, the animal or pair of animals is associated with its identifier. This technique has had some failures when the cage marks fade or are rubbed off, or the attached card is removed. This deficiency can be overcome by simple maintenance of the numbers and cards.

INVASIVE TECHNIQUES

Freeze/Heat/Chemical Branding

Skin branding is the process of causing a scar to form on the surface of the skin in a manner that makes an identifiable mark. This can be done with heat (direct heat branding or electrocauterization) (Clark Jr, 1971) freeze branding using liquid nitrogen or dry ice (Daugherty, 1976; Paine et al., 1984; Measey et al., 2003); or by using chemicals (using a solution of 0.5% amido Schwartz in 7% acetic acid)(Wolf and Hedrick, 1971). This method is painful; and therefore, requires local or general anesthetic. In addition, due to the nature of amphibian skin these marks are only semi-permanent in some species (CACC, Unknown).

Freeze branding has been employed in field studies for larger species of amphibians such as Cryptobranchids. The animal is “branded” with a mark or number using extreme cold. A metal branding tool is cooled to well below freezing with a refrigerant or dry ice. The branding tool must be of sufficient mass to effectively freeze the skin and pigment cells of the animal. When the very cold brand is placed on the wet skin of an amphibian for a few seconds, the tissues in contact with the brand will freeze and die. The resulting area usually heals without pigment, making a permanent mark on the animal. There are disadvantages to this method. First the brand itself is unaesthetic and visible. Second, the method is crude and the brands are usually large. The actual area frozen by the brand is not easily controlled. The longer the brand is in contact with the skin, the wider the brand. The difference of a small acceptable brand and a large area of scar tissues may be the result of a few additional seconds of contact time between the amphibian and the branding tool.

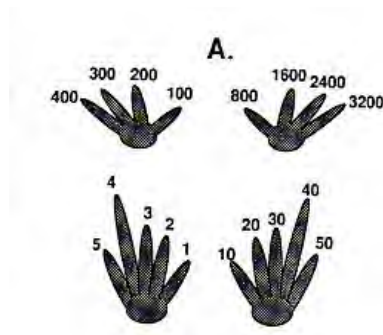
Though the freeze branding marks have lasted a relatively long time in large species such as cryptobranchids, there may be limits in smaller species. Both heat and freeze brands have been used in toads. Clark (1971) describes a heat branding method in which wire is formed into the shape of numbers and used to apply unique marks to 311 Gulf Coast toads (*Incilius nebulifer*) with mark retention of a year. Paine et al. (1984) tested freeze branding on Puerto Rican crested toads (*Peltophryne lemur*) at the Buffalo Zoo with success and marks lasting over 2 years.



The photograph to the left shows a freeze brand on a fish; in this case, a single line. It is easily seen here that there are limitations to the number of variations with this crude mark.

Tissue Amputation

Toe clipping has been a common technique in field research projects for reptiles and amphibians for many years. This technique is invasive and creates a permanent deficit in the animal. It has the potential to increase mortality (Clarke, 1972). Still, this is a technique that has been employed in some zoo programs. The technique involves amputating digits from the animal that corresponds to a numbering scheme. Below is an example of a number system for up to 10,000 individuals (Twitty, 1966).



From Donnelly (1994)

A problem with this method is that it may require the removal of multiple digits from a single appendage, leaving the animal little more than a stump. This can compromise the

competence of the animal to perform simple essential biological functions such as locomotion, breeding embraces, feeding, etc. In addition, rare infections are noted at the amputation site.

One place that this technique has been used is for some conservation release programs. Usually young amphibians are monitored by groups and are all given a single mark coding (i.e., the removal of one digit). Older animals may be marked with an individual code. The use of this technique has to be weighed carefully between the need to track animals and to obtain release survival rates and the potential harm that may be done to the released animals.

In addition, toe regeneration was an issue in some species, especially salamanders (Davis and Ovaska, 2001), so Heatwole (1961) used beryllium nitrate to inhibit the regrowth of the toes in *Plethodon cinereus*, which was very successful for the study. Heatwole acknowledged that beryllium nitrate is known to be toxic and cause edema and death at even low concentrations, but used a dilute concentration carefully applied to the clipped toe (Heatwole, 1961). American Society of Ichthyologists and Herpetologists (2004) recommends limiting the number of toes clipped per animal and avoiding removal of two adjacent toes (Beaupre et al., 2004). May (2004) wrote an article stating that the use of toe clips statistically compromised of studies using these marks.

As molecular ecology and skeletochronolgy have become tools in ecology, toe clipping is often a marking method where the removed tissue has important uses. Lien (2007) recently used toe-clipping to determine the demographics of Taipei Grass Frog (*Rana taipehensis*) and used the toes for skeletochronological investigations. If toe clips are used, all removed toes should be cataloged and stored in an appropriate manner for possible analysis, especially in rare or endangered species.

Tail clipping in salamanders has also been used as a marking technique; however, the regenerative ability of salamanders makes this a temporary method at best. Arntzen et al. (1999) compared marking and tissue sampling methods in the newt *Triturus cristatus* and recommend it even though the clips grew back within about eight months. This is another opportunity for researchers to collect genetic samples. Tail clipping in tadpoles has limited utility as well since the mark is generally lost within 2–3 weeks of amputation (Turner, 1960). Guttman and Creasey (1973) noted that tail clipping also has the risk of causing damage to blood vessels or nerves.

Passive Identification Transponders

Passive identification transponders (commonly referred to as PIT tags) are perhaps the most common method of identification for zoo animals. A microchip is place under the skin in the back or into the coelomic cavity of the animal. This is accomplished by making a small incision in the animal and manually placing the tag, or by using an applicator. The applicator is a large hypodermic device with a 12 gauge needle. The tag can then be detected by a reader that sends radio signals to the transponder, which excites a transmitter in the tag to return a unique alpha/numeric code that is decoded by the reader. There are billions of codes available that assure that the codes are unique. The code appears on a screen in the reader.

This is an invasive technique that potentially exposes the animal to infection, although this has rarely been reported. There are also limitations. The tags are ~1.2cm long and 2.2cm wide. Frogs must be at least 25–35 mm in SV length to accept the tags. Also, the tags frequently migrate in the body of the animal and it is not uncommon for the PIT tags to be expelled from the amphibian, usually from a different location than the original insertion. This is particularly true when the tag is placed in the body cavity. Tags are known to be passed in the feces. When the tag is expelled, the identification of the animal can be lost.

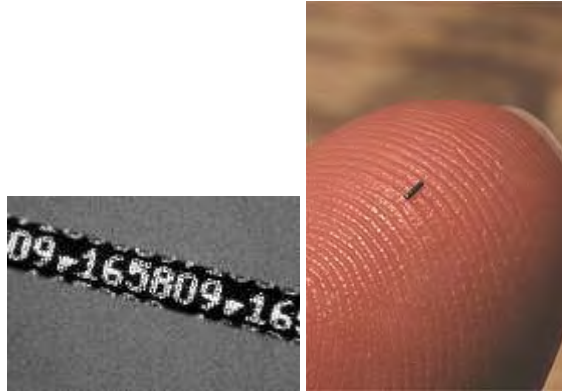
Another issue with PIT tags is that there are several systems on the market which are incompatible. The most common tags are sold by AVID and Trovan. There has been a patent dispute between these companies and AVID tags may be more easily obtained in the U.S. An AVID tag cannot be read by a Trovan reader. AVID does make a universal reader that will read both tags and another European system, but it is expensive (~\$1,200 USD). Some programs have adopted Trovan tags as a standard and some AVID. This may make it necessary to have more than one system available at your institution.



Portable Avid Transponder Reader

Decimal Coded Wire Tags

A technology developed for the fisheries managers to monitor released fish is to use small sections of magnetized wires with tiny (micro) numbering imprinted on the wire (Donnelly et al., 1994). The presence of the wire is detected with a magnetic detector system. It then must be removed in order to read the number with a magnifying device. The removal of the tag is a surgical procedure and there is risk for mortality, functional deficit, or scarring. This technique has limited applications in captivity, but could be a valuable tool for release programs where large numbers of small animals are released.



From : <http://www.nmt.us/products/cwt/cwt.htm>.

Visible Implant Fluorescent Elastomer (VIE) tagging system

Another technique using pigmented polymers also has its origins in fish studies. The technique involved injecting a visible fluorescent elastomer subcutaneously into the animal (Donnelly et al., 1994). The elastomers are available in a variety of colors. Some colors of the elastomer can then be seen by placing the animal under a black light, other do not fluoresce and must be visible without the light for identification. The fluorescing dyes are clearly visible under the skin even with some pigmentation. By placing the elastomer at different sites on the animal and using different colors, an animal can be identified. Below is an example of marking of a frog using the webbing in the hind foot.



From: <http://tropicalis.berkeley.edu/home/husbandry/tags/E-ANTs.html>

Systems have also been developed for salamanders. The photo below shows four sites that can be used to code identification with various colors.



From: <http://www.pwrc.usgs.gov/resshow/droege2rs/salmark.htm>

Another application for elastomers is to mark larvae. The elastomer becomes a permanent part of the animal. When the animal metamorphoses into the adult form the elastomer tag can remain visible. One problem with technique is that you never really know where the implant will end up on the metamorphosed animal. Tissues move and proliferate during the metamorphosis process. Tagging of larvae often requires that the animals are anesthetized, especially in smaller species, which carries its own risks. The small size makes it impossible to safely and effectively restrain the larvae while tagging. With anesthesia, very small animals can be marked. For example, larval red spotted newts were successfully marked at the Detroit Zoo and retained their tail tags.

Another issue is that some of the elastomer may migrate in body of the animal. In Wyoming toads, it was discovered that elastomers injected in a leg could be detected under UV light in the liver when the animal was later examined during necropsy (Williams, 1995). Due to the skin of amphibians not being directly adhered to the muscle, tags placed subcutaneously will often migrate to the lower part of the body. Multiple color tagging of frogs in the thighs often results in a collection of color spots in the groin area. This can be resolved by injecting the elastomer into the surface of the underlying muscle. Once injected, gently running your finger over the site will indicate if the tag is secure.

The use of elastomer has been compared to other methods of marking, including Davis and Ovaska (2001) who compared elastomer tagging to toe clipping. They found that western red-backed salamander, (*Plethodon vehiculum*) tagged with elastomer showed better weight gain than animals that were toe clipped in the lab and in the field. Heymeyer et al. (2007) tested elastomer in eastern red-backed salamanders (*Plethodon cinereus*) which have dark skin which could limit the value of this technique.

Their results indicate that this marking technique is a viable option though there was some migration of the tags and they therefore recommend placing multiple marks as far away from each other as possible (Heemeyer et al., 2007). The manufacturers of this product site a number of papers that have tested the applicability of this product including Register and Woosley (2005) who used VIE to identify and track the egg masses (Northwestern Marine Technology, Application Note APG02, 2007).

Other Injectable Color Markings

Over the years, a variety of methods involving the injection of a highly visible product into the animal have appeared. All these methods were initially applied to the fisheries industry where they were refined and sometimes even automated. The techniques used most often appear to be Panjet™ (Wright Health Group, Ltd. Dundee) and other tattooing methods, injectable acrylic polymers, and Visible Implant Elastomer (which we have discussed) (Northwestern Marine Technology, Salisbury UK).

Tattooing in general implants some kind of dye into the skin at a depth to avoid the coloration washing off. The Canadian Council on Animal Care (CACC, Unknown) manual for amphibian and reptile care recommends selection of a tattooing method based on 1) use of a dye that contrasts with the animal's skin and 2) use of a tattoo that maintains legibility over time, with diffusion into the skin, and in ultraviolet degradation (Canadian Council on Animal Care, 2005). However, even with these considerations Murray and Fuller (2000) recommend using tissue removal, branding, freeze branding, and electrocauterization over tattooing due to the potential for problems with visibility and legibility. Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists (Beaupre et al., 2004) approves of the use of tattooing as a marking method, but cautions that the dye being used has the potential for absorption and, if the toxicity is unknown in amphibians, possible deaths. In addition, the permeable nature of amphibian skin makes a tattoo marking prone to diffusion.

Panjet™ tattooing is an automated method of injecting dyes intracutaneously through pressurization instead of using a needle. This process is commonly used in fisheries as the small aperture and high pressure of the device essentially forces the dye into the skin of the animal. Measey (2003) used this technique in caecilians and found the marks to be reliable and, based on observation only, did not impact the survival or behavior of the animals.

Though the name brand Panjet™ was not mentioned, Nishikawa and Service (1988) used a high pressure, needless method like it in a comparison of this technique and toe clips for recapturability in the salamanders *Plethodon jordani* and *P. glutinosus*. They altered the previously used method by decreasing the size of the aperture of the gun using a small tube and placing marks in various locations on the body and limbs. The results of this study showed that this marking method was successful, with results better than those of the toe clipped animals for recapture (Nishikawa and Service, 1988). Taylor and Deegan (1982) studied the effectiveness of this method in green frog (*Lithobates clamitans*) tadpoles and found it to be successful in marking large numbers of larva, however, they do warn that the pressure may be an issue if trying to mark

small, delicate animals. They also did not investigate the possible impacts of marking on larval growth (Taylor and Deegan, 1982).

Another injectable marking protocol used in a variety of amphibian studies is injectable acrylic polymer. Wooley (1973) used this method in salamanders with success, though individual markings were not possible. This process gave marks that were visible from 4–5 feet away; however, there was some slight fading along the perimeter of the mark and slight instability of the marks over time in a few animals. The main advantage noted by Wooley was the ability to observe the animals without capture and handling after the initial marking. However, increased visibility to the researchers would imply increased visibility to predators which may impact survival. Cecil and Just (1978) used the same procedure to mark larval bullfrogs (*Rana catesbeiana*). Though there was some difficulty discriminating similar colors (such as white and yellow) the method was successful and cost effective overall (Cecil and Just, 1978).

Marking larval amphibians presents a unique challenge due to their size and structure. Seale and Boraas (1974) used mixture of dye, petroleum jelly, and mineral oil and injected it into the tail and back of the tadpoles. They found the markings to be permanent (until metamorphosis) and have no impact on the animals. If not placed correctly, however, there were problems with the swimming motion of the tad forcing the mark out and would therefore require remarking. Others had much less success with this method since the ratio of mineral oil to petroleum jelly must be exact, and there is a lack of consistency in the available products to allow for regular success.

Larval Dying

Dying whole tadpoles has been used in a variety of studies over the years. In all these methods, the main variable is the dye used. Regardless of the dye, a solution is made and the tadpoles are placed in the dye solution for a set amount of time. The dye absorbs into the semi-permeable skin of the larvae and colors the whole animal. Guttman and Creasey (1973) found that methyl blue killed tadpoles and stained internal organs, neutral red only lasted two days, Bismarck brown Y caused sluggish behavior. If the purpose of the study is to understand survival and behavior in larval amphibians, the temporary effects of neutral red appears to be preferable. Travis (1981) used neutral red to stain *Hyla gratiosa* tadpoles and assess the impact of dying on tadpole growth and survival. Their data indicates that dying tadpoles decreased the growth of the tadpoles. This should be a consideration in the decision process for considering its use.

Visible Alpha-Numeric Tags

Visible alpha-numeric tags that can be applied to amphibian identification are commercially available (Donnelly et al., 1994). These tiny tags are inserted subcutaneously by making a small incision and placing the tag under the skin. Closure of the skin is performed using surgical glue (cyanoacrylic glue, e.g., Crazy Glue). One recommended site for implantation is the inside of the thigh on frogs (see

<http://tropicalis.berkeley.edu/home/husbandry/tags/E-ANTs.html>). These tags are read by using a special blue LED flashlight and amber viewing glasses available from the manufacturer. The tags come with alpha-numeric numbers that provide about 46,000 unique variants. These tags are printed with unique numbers and come in two sizes, (standard at 1.0 mm x 2.5 mm and large at 1.5 mm x 3.5 mm) and in a variety of colors. Workers (Measey et al., 2001; Measey et al., 2003) used this method successfully in the caecilian (*Gegeneophis ramaswamii*) which have previously only be individually identifiable in small captive groups where the annulations pattern was distinguishable. This method did require anesthesia due to the overall difficulty in handling legless amphibians, but would not require general anesthesia in species that were easily restrained.



From: <http://tropicalis.berkeley.edu/home/husbandry/tags/E-ANTs.html>

Bands and Tags

Some older field studies used cords around the waists of frogs as marks. Some of the cords were color coded while other held small tags. The Canadian Council on Animal Care sites Bull (2000) for comparing waist and arm bands and finding both to cause abrasions in frogs (CACC, Unknown). Raney (1940) sites a study by Breder, (Breder Jr et al., 1927) using a cord with a small tag and his concerns were the lack of permanence and the potential for injury when the cord was tied too tight Raney (1940). However, Lien (Lien et al., 2007) successfully used waist bands for individual identification in Taipei Grass Frogs (*Hylarana taipehensis*), so there are current uses for this method.

Generally, waist bands, with or without tags, appear to have a limited life span and have the potential to snag of items and potentially inhibit movement. In addition, the colors of the bands may attract predators and we have found no studies on the possible implications to survivorship. However, as a temporary marking technique in captive animals, it may be useful and viable.

Bands around the forelimb were apparently used by Dely (1954) in *Rana esculenta* and *Bombina bombina* according to Honegger (Honegger, 2007). Honegger was primarily interested in marking techniques for zoos and; therefore, dismissed this marking technique due to its aesthetically displeasing characteristics rather than

practicality and utility. The other concern with this marking was that it was only seasonal. Depending on the material used to construct the bands, there is also risk of abrasions or injury, interference with amplexus, and attraction of predators. The forelimbs of many male anurans are robust and would not lend themselves to having a band stay in place easily without injury.

Honegger (2007) also refers to a study where bands were placed around the digits of amphibians. This was a temporary marking method performed on an unidentified frog species by Dely (1954). We were not able to find the original paper, but it would seem that the anatomical structure of a frog's foot would make tags easy to lose, and if placed tight enough to hold in place, would cause strictures which could result in the loss of toes. In addition, any of the bands could interfere with shedding in amphibians as well, depending on how they are applied.

Knee tags were used by McAllister et al. (2004) in a comparison in marking techniques with radio telemetry. He cites the use of these tags by Elmberg (1989) using elastic cord. The tags were plastic, numerically coded fingerling tags tied at the knee. They found the tags to cause skin irritation and lacerations of the skin and muscle.

As part of a small (2 frog) demonstrational telemetry project, the Detroit Zoo placed transmitters on adult male American bullfrogs (*Lithobates catesbeianus*) using a small chair waist band covered in plastic. Though the tags were eventually lost, no injury was noted on the recaptured animal prior to the loss of his tag. Banding may be more applicable to captive management as the animals are monitored daily and a single band loss could be resolved easily.

External Tags

We distinguish external tags from bands because tags generally involve passing some part of the marker through the skin and/or muscle of the animal. Tags are more invasive and have a potential for greater discomfort for the animals, injury, and infection; however, they do have a higher degree of permanence and reliability. There have been a wide variety of external tags used in amphibian studies over the years. Some of these include jaw tag (Raney, 1940; Raney and Lachner, 1947), tail clips (Raney and Ingram, 1941), bead tags (Nace and Manders, 1982), knee tags (McAllister et al., 2004), and toe tags (CACC, Unknown). Researchers studying amphibians 50–70 years ago appeared to show less concern for the comfort of the animal during the placement of the tags and over the life of the animal with the tag, though its comfort was not completely ignored. The attitude of researchers regarding humane placement of marks is evident with a review of the literature. In addition, recent researchers are required to view test animals in the same light as mammals and birds for permitting. Amphibians were, until recently, classified with the fish by universities and labs, which are not as regulated for testing and handling.

One of the earliest accounts of using a tag was Raney (1940) who used jaw tags on American bullfrogs (*Lithobates catesbeianus*) and green frogs (*Rana clamitans*). During the study he marked 606 frogs using numbered metal tags placed through the lower jaw. This is a method that was used on fish, and Raney's dissatisfaction with toe clipping (due to bookkeeping) and waist bands (due to permanence) led him to use the

jaw clips in frogs. He started with 50 frogs as a test group since he was concerned about the implications of the tags on the frogs' survival and behavior. This procedure was done with no anesthesia and Raney reports some of the frogs pulling on the tags with their feet after insertion but "no serious tearing of the flesh was noted". He claimed there was not apparent impact on the animal's ability to eat since the stomachs of recaptures contained food upon palpation and that the call was not noticeably changed. He concluded that the jaw clip method was satisfactory and superior to the other methods available at the time (Raney, 1940). Between 1940 and 1946, Raney and Lacher investigated the impact of the jaw clips on the growth of the toad (*Anaxyrus terrestris americanus*) (Raney and Lachner, 1947). They found that the tagged animals had a slower growth rate than the untagged animals. Woodbury (Woodbury et al., 1956) cites a study by Stebbins, et al. (1954) who found jaw clips to be an unacceptable method of marking salamanders. They found the tags caused inflammation and were slough off through the jaw bone. Recent use of jaw clips is nonexistent from the literature for apparently good reasons.

Tail clips on salamanders were attempted by Raney (1941) where he attached metal bands through the tail and back, however these marking methods failed. In some cases the clips that he used were so heavy that aquatic newts had difficulty resurfacing in water. He also saw a large number of lost tags, tail injuries, at least on completely dropped tail, and what he called "putrid" skin, which we assume to mean an infection of some type. Raney does, however, mention that another researcher used tail clips in *Necturus* with success during the period of the study (Raney, 1941). Overall, using tags on salamanders have been less than successful and are not recommended.

Nace and Mander (1982) created a unique marking system for captive *Xenopus* which utilizes a surgical wire with colored beads stung onto it. The wire is placed through the fore or hind limb around the bone to assure its stability. In this study, the female frogs were marks on the forelimb and the males on the hind limb to avoid complications and snagging during breeding. Tags placed only through the skin were occasionally lost (Nace and Manders, 1982). Using bead wire tags through the thigh or tail of amphibians is also possible, though the risk of snagging does exist (CACC, Unknown). In larger frogs, toe tags are an option for marking animals. These tags are placed through the webbing of the hind foot and include a disc-type tag which contains a unique code (CACC, Unknown). Honegger (2007) refers to the use of toe tags by Heusser (1958) who encountered swelling at the site and retention of about one season, with a maximum of two seasons.

CONCLUSIONS

There are many options for maintaining the individual identity of an amphibian through its life. Many have been employed in field work and later adopted for captive animals. In captivity the animals are monitored continuously; unlike many animals in field studies that are marked and then released into the wild. If there is an impact on survivorship or morbidity caused by a marking technique it can be directly observed for the entire life of the animal. For long lived taxa this can be decades. Modern veterinary

science and pathology can now identify issues from marking techniques that have not been evident in the past (Williams, 1995). This has provided additional information on the suitability of some techniques.

Today current animal ethics limit the options available for zoo animals. Considerations for animal welfare, specimen aesthetics, and long-term health of the specimen are essential. Non-invasive techniques are preferred if they are reliable, functional, and workable. When the techniques are invasive, it is vital to balance the benefits of the technique (i.e., animal pedigree) and the potential costs in pain, morbidity, fecundity, and mortality to the specimen.

Mutilation through tissue amputation is considered an unacceptable technique at some institutions. This is particularly true when there are multiple amputations involved in the marking. These techniques can limit function, create the opportunity for infection, reduce functionality, and increase mortality. Tissue regeneration is also clearly evident if it occurs to captive animals. The pros and cons with some comments for captive situations of many of the techniques described in this chapter are provided in Tables 1 and 2 below.

R. Andrew Odum
Department of Herpetology, Toledo Zoological Society
raodum@aol.com

Edythe Sonntag
Michigan State University
sonntage@msu.edu

TABLE OF PROS AND CONS OF MARKING TECHNIQUES

TABLE 1— NON-INVASIVE TECHNIQUES

Technique	Application	Pros	Cons
Photographic ID	Animals with unique patterns or structures (i.e., warts)	Noninvasive	Requires some photographic expertise. Patterns can change with age. Postmortem changes may make it difficult to identify the animal
Pattern drawings	Animals with easily identifiable different patterns	Noninvasive	Requires some photographic expertise. Patterns can change with age. Postmortem changes may make it difficult to identify the animal
Isolation	Animals are held individually or in pairs (sexually Dimorphic)	Noninvasive Does not require pattern	Cage labels or cards may be lost

Table 2 – Invasive Techniques

Technique	Application	Pros	Cons
Freeze Branding	Large Amphibians	Permanent marking	Markings are crude Can cause infection Animals must be fairly large
Toe Clipping and tail clipping	Frogs and Salamanders	An easy technique	May require multiple digit amputations on one limb. Salamanders may regenerate digit and tail Can cause infections
Other amputation	Not recommended	An easy technique	Leaves mutilation May compromise function
Decimal Code Wire	Groups of animals	Can be used to mark many animals	Requires reader Animal may have to be euthanized to read wire
Bands	Frogs and salamanders	An easy technique	Cannot be used on small animals Tag may fall May compromise function
External Tags	All amphibians	Easy Id	Cannot be used on small animals Tag may fall May compromise function
Passive Integrated Transponders	Animals above 25g mass	Unique ID and common use in zoos	Cannot be used on small animals Tag may be shed Tag may fail
Visual implants - Elastomer	Marking of any amphibian	Can be used on larvae and adults	Elastomer may migrate to internal organs Limited coding systems Requires black light
Other Injectable Implants	Marking of any amphibian	Can be used on larvae and adults	Elastomer may migrate to internal organs Limited coding systems Requires black light

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Appendix 2

WASTEWATER TREATMENT**Design considerations for wastewater treatment**

To accomplish effective wastewater treatment both biological and chemical methods can be used. Under certain circumstances the on-site processing of water is necessary. To determine the need and appropriate type of effluent treatment, risk assessment for introducing novel pathogens to the environment is necessary. Consideration should also be given to toxic chemical releases from these facilities.

- In a situation where wastewater treatment is deemed necessary on site, the system ideally must provide uninterrupted service, require little maintenance, and have minimal operating costs. As an example, if we consider that there is a high probability that animals held in a facility may carry a virulent pathogen that could harm local amphibians, system efficacy and reliability are primary concern. Systems need to be tested by culturing effluent to determine their efficacy. Even if a system is 99% reliable, it means that it would be down for 3.65 days per year and pathogens would be released into the environment. If there is truly a dangerous pathogen present, this would not be considered acceptable.
- In assessing different disinfectants for water treatment, it is necessary to have some metric for its efficacy against specific organisms (pathogens). This efficacy is normally defined for a percentage killed (i.e., 99.99%). A standard metric that has been commonly used is the product concentration and contact time (Ct) (Ct = concentration in mg/l (ppm) and contact time in minutes). Ct concept allows you to determine the amount of contact time needed at different concentrations. For instance, a Ct of 100 with a 99.99% kill for a pathogen would be effective if the contact time was for 100 minutes at a concentration of 1.0mg/l or for 1 minute at a concentration of 100mg/l. Ct varies widely for different disinfectants and different pathogens. It is also temperature dependent. Disinfectants are generally more effective at higher temperatures. Ct is used as the standard by the U.S. EPA for wastewater treatment and there is significant data to support its use as a standard for amphibian pathogen control.
- Ct is pathogen specific. The Ct for free chlorine is ~ 100 for *E. coli*, and as high as 10,000 for *Cryptosporidia parvum*. *Batrachochytrium* (Bd) has a similar Ct to some of the more chlorine resistant pathogens such as *Giardia* and *Mycobacterium* (Ct = ~1000mg-min/liter).
- There are also biological systems that have demonstrated efficacy in controlling the spread of pathogens. These include local septic systems commonly used for homes and larger wastewater treatment facilities that use biological processes with a final disinfectant applied before final release of the effluent.

Septic systems

An option that provides a reasonable level of disease control is a septic system that includes a settling tank and a seepage field that distributes wastewater under the ground. These systems have demonstrated their ability to control the distribution of pathogens into ground water and the environment. This is clearly indicated in the standards for potable water wells and septic field discharge in the United States. The standard is that a distance between the well intake and septic field must be a minimum of 100ft apart. This distance is sufficient to prevent pathogens from entering a potable water system. A deficiency in these systems is that they do not control non-biodegradable chemicals that can enter the ground water. The settling tank also needs periodic maintenance to remove built up sediment.

Suspended matter in waste water

The first step in any water treatment process is the removal of chemical contaminants. This can be accomplished by using mechanical filtration or settling tanks. Most chemical disinfectants are ineffective in treating wastewater that contains suspended particulate. Depending on the particulate composition, the chemicals may not penetrate, allowing pockets of pathogens to escape disinfection. This creates another disposal issue for the residue, a solid waste (see Solid Waste below).

Chlorine (Cl₂)

- Free chlorine (Cl₂) has commonly been used as a disinfectant for amphibians. The most common source of free chlorine is sodium hypochlorite (NaClO) solution. It is readily available as household bleach (3-6% NaClO) and commercial preparations with concentrations as high as +30%. Another common source of free chlorine is calcium hypochlorite Ca(ClO)₂. The latter may be sold under a variety of names including HTH for swimming pool chlorination. Ca(ClO)₂ is a solid which may contain as much as 50% free chlorine. Because of its higher concentration of free Cl₂, it is an ideal choice for treating large volumes on water.
- General concentrations as high as 1% NaClO (i.e., 1 part of 4% bleach to 3 parts of water) for cleaning have been recommended for cleaning and disinfection (Pessier, 2008). The free chlorine in this dilution is ~47,600mg/l. These high concentrations are impractical for large water treatment systems.
- Chlorine is a viable option for disinfecting wastewater for some organisms. Unfortunately if Bd is the primary concern for isolation, experiments by Johnson, et al. (2003) demonstrated that it is very resistant to chlorine disinfection. In their experiments, 100% of Bd was killed with a concentration of 4,00mg/l NaClO (=0.4%, 1 part 4.0% bleach to 9 parts effluent) for a contact time of 10 minutes. It was also killed with a concentration 10,000mg/l in one minute (=1.0%, 1 part 4.0% bleach to 3 parts of water). This establishes Ct between 10,000 and 40,000 mg-min/liter. Many institutions employ manual disinfection of water using bleach. For small amounts of water this can be practical using the 1:30 bleach-wastewater dilution with contact time of 40 minutes (or 1:9 dilution for 10 minutes).

- For larger volumes automated techniques would be advantageous, but again chlorine has its deficiencies for Bd. A system has been developed at the Perth Zoo designed around readily available chlorination system components for swimming pools (Robinson, et al., 2008). This system holds water in a reservoir and automatically injects a NaClO solution with a chemical pumping system. The concentration of the chlorine in the reservoir is controlled by a chlorine sensing probe and analyzer. The concentration in the reservoir is maintained at 100mg/l. If we consider Ct at its upper limits of 40,000mg-min/l, the contact time would have to be 400 minutes (6.66 hours). The efficacy of this system has not been valuated with live Bd, but it seems promising.

Heat treatment

Raising the temperature of the water to boiling is generally considered a good method of disinfecting. Unfortunately it is also very expensive. Water has a very high specific heat and it requires a great deal of energy to raise it to boiling temperature. Thus systems designed for large amounts of water would be cost prohibitive to operate. However, this is a viable option for disinfecting small quantities of wastewater.

Ozonation

Ozone is a very strong oxidant and one of the most effective disinfectants available. No specific tests have been done on Bd, but there is no reason that it should not be effective at normal concentrations. The Ct for difficult to kill pathogens such as *Mycobacterium* and *Cryptosporidium* are two orders of magnitude less than those demonstrated by Chlorine (Ct = ~10). Another benefit of Ozone is that it rapidly breaks down into non-toxic oxygen, resulting in no residual left in the environment.

Below is a schematic of a simple wastewater treatment system using ozone and UV light. Systems of this type can prove effective in wastewater disinfection. Experiments with real amphibian waste water have provided promising results, but further refinement is still necessary to develop a design that could be used widely.

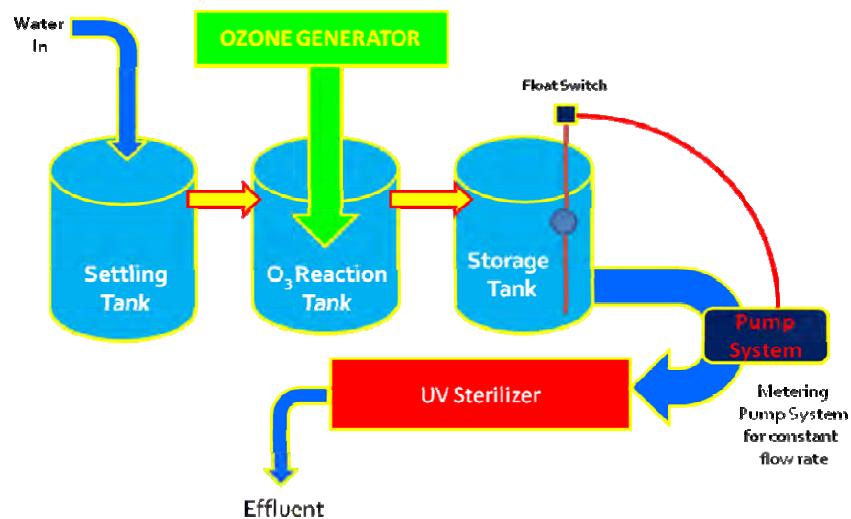


Illustration by R. A. Odum

The disadvantage of ozone is that it is expensive to produce, requires substantial initial investment, and requires considerable maintenance. The highly corrosive and reactive properties of ozone require that all tubing, tanks, and piping are made of ozone resistant materials.

UV light sterilization

UV sterilization has been employed for years for water treatment. For it to be effective, most particulate must be removed before treatment. Unfortunately the one pathogen that seems to be very resistant is Bd. Still, Bd is not the only pathogen that is associated with amphibians and UV can be used in conjunction with other methods to produce a more effective system.



Appendix 3

ISOLATED AMPHIBIAN ROOMS AT OMAHA'S HENRY DOORLY ZOO

Reprinted, with permission, from: Zippel, K., R. Lacey, O. Byers (eds.) 2006. CBSG/WAZA Amphibian *Ex Situ* Conservation Planning Workshop Final Report. IUCN/SSC Conservation Breeding Specialist Group, Apple Valley, MN 55124, USA. Pp. 65.

ISOLATED AMPHIBIAN ROOMS AT OMAHA'S HENRY DOORLY ZOO

An Example of Complying with the Quarantine and Husbandry Standards for Amphibians Designated for Reintroduction into the Wild

Jessi Krebs

Supervisor of Reptiles and Amphibians, Omaha's Henry Doorly Zoo

3701 S. 10th St.

Omaha NE 68107

jkrebs@omahazoo.com

Photos by J. Krebs

INTRODUCTION

In February 2006, the IUCN Conservation Breeding Specialist Group (CBSG) and the World Association of Zoos and Aquariums (WAZA) hosted an Amphibian *Ex Situ* Conservation Planning Workshop in El Valle Panama. One of the many purposes of the meeting was to make recommendations for husbandry standards for amphibians that are part of reintroduction programs or captive collections that may be returned to the wild at some point in the future (Zippel et al., 2006). Many of the recommendations

made involve upgrading current housing and quarantine standards practiced by many zoological, private, and academic institutions, and have been seen by some as impractical and extreme for zoos and aquariums. Armed with the lessons learned from the global spread of *Batrachochytrium dendrobatidis* (*Bd*; the amphibian chytrid fungus) and the potential for new pathogens to emerge, it would be prudent for institutions that are housing amphibian species designated for repatriation to review their basic husbandry practices and quarantine standards, attempting to comply with the new recommendations.

Omaha's Henry Doorly Zoo responded to this 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. The following list of images, prices, materials, and sources are provided to serve as an early example to others who might consider constructing their own IAR facilities for amphibians.¹



Figure 1. A typical non-compliant amphibian room.

AMPHIBIAN ROOMS

Not Biosecure Compliant

Most amphibian-holding rooms at zoos and aquariums are not in compliance with new bio-security recommendations. One example of a non-bio-secure amphibian room is one that houses animals from all over the world (Figure 1). Other problems may be that steps were not taken to prevent wastewater from spilling from tanks placed on higher shelves into tanks below, or a wastewater treatment process was not employed to prevent pathogens from exiting the facility and endangering local amphibian populations. Keeper error can never be completely ruled out, and unsecured lids may further increase pathogen spread between animals from different areas of the world.

¹ Materials and sources cited are presented based on fabrication at Omaha's Henry Doorly Zoo, not as an endorsement. Contact the author for additional information about any of the products presented.



Figure 2. An example of the Isolated Amphibian Room – size 8 x 8 x 8 ft (2.4 x 2.4 x 2.4 m)

Biosecure Compliant

Each IAR at Omaha's Henry Doorly Zoo holds just one species or one species assemblage from the same area. The IARs are versatile rooms constructed out of commercially available greenhouse materials with all construction (Figure 2). IARs at the zoo range from 8 x 4 x 8 ft (2.4 x 1.5 x 2.4 m) in size to 10 x 16 x 8 ft (3 x 4.9 x 2.4 m). The walls are made of 1.5 x 1.5 inch (3.8 x 3.8 cm) hollow-aluminum tubing overlaid with two-ply Lexan® sheeting. Individual walls are joined together with 1 inch (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 the room in Figure 1 above:

Cap² 18 @ 8 ft (2.4 m)
 Splice² 3 @ 8 ft (2.4 m)
 Lexan^{®2} 6 @ 6 x 8 ft sheets (1.8 x 2.4 m)
 Aluminum Tubing³ 18 @ 8 ft [1.5 x 1.5 inch (3.8 x 3.8 cm);
 1/8 inch (0.3 cm) thick]
 Storm door
 Hardware
 Screws
 Washers n completed by zookeepers

² www.stuppy.com

³ www.statesteel.com/omaha.htm



Figure 3. Close-up of the 1inch (2 cm) aluminum angle pieces holding the 1.5 x 1.5 inch (3.8 x 3.8 cm) aluminum tubing and storm door.



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

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: 8 x 8 x 8 ft (2.4 x 2.4 x 2.4 m) rooms use 10,000 BTU units; the 10 x 16 x 8 ft (3 x 4.9 x 2.4 m) use 12,000 BTU units. 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.

List of items used in the room shown in Figure 4 above:

Heater/AC⁴ 10,000 BTU units

⁴ www.sunpentown.com/wa12pocwihe.html

Footwear



Figure 5. Shelving with amphibian enclosures.

Tubs used for amphibian enclosures are made 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 gallons to 16 gallons.

List of items used for the shelving within the room shown in Figure 5:

- Shelving units⁵
- Frog tanks⁶
- Lids⁷

⁵ www.samsclub.com/shopping/navigate.do?dest=5&item=203424&pCatg=7085 or from materials acquired at local hardware stores

⁶ www.rcpworksmarter.com/rcp/products/detail.jsp?rcpNum=3328

⁷ www.habitatsystemsllc.com, custom fabricated

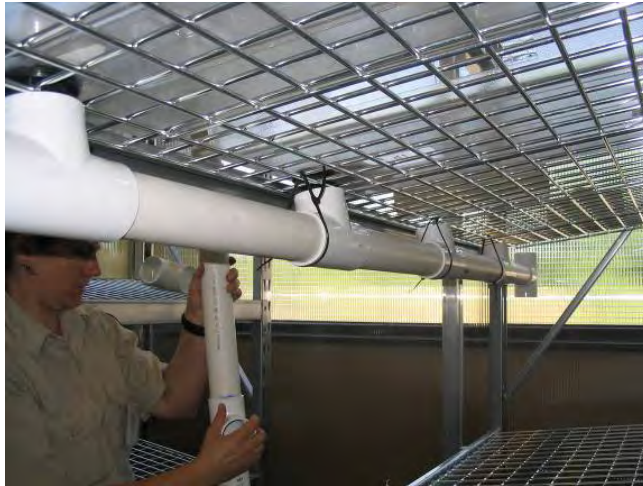


Figure 6. The over-sized drain system under each shelf being installed in the IAR.

The drain for each enclosure runs into a common drain system located under every shelf. Drain system lines are 2-inch (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).



Figure 7. IAR wastewater collection tub with sump pump below.

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 with an automatic on/off switch is set within the lower tub to pump wastewater to the Central Treatment Station (Figure 8). 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.

List of items used for the wastewater collection tub in Figures 6 and 7 above:

Two utility sinks
 Sump pump⁸
 PVC pipes, T's, and elbows
 Plumbing



Figure 8. The building's water storage and central treatment station.

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 8; See Chapter 1 for additional information on reconstituted RO water). Two barrels are used to collect all wastewater (center of Figure 8), which is then treated with household bleach for 12 hours before being released into the city sewer system. See Chapter 3 for more information on wastewater treatment.

List of items used for influent and effluent water treatment within the room shown in Figure 8 above:

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	
PVC piping	
Plumbing	
Water quality test kits	

⁸ www.flotecpump.com/pdf/Page_06_2004.pdf



Figure 9. Lighting accommodated into the racks.

Lighting on every rack system is provided in two forms: compact florescent lights above each shelf to provide ultraviolet light and small heat lamps on each enclosure to provide basking sites for species requiring higher temperatures (Figure 9).

List of items used for lighting in the room shown in Figure 9 above:

Lighting fixtures⁹

Bulbs¹⁰

Summary Budget for an 8 x 8 x 8 ft (2.4 x 2.4 x 2.4 m) IAR:

Room materials	1,100 USD
Shelving	270 USD
Heater/AC	700 USD
Frog tanks	145 USD each x 18 = 2,610 USD
Lighting	210 USD each x 9 = 1,890 USD
Plumbing	450 USD
Electrical/duct work	200 USD
TOTAL for one room	7,220 USD

CONCLUSION

The Johannesburg Zoo of South Africa has used similar technologies and practices to develop isolated amphibian rooms in their efforts to meet international biosecurity standards at their Amphibian Conservation Center. At the Johannesburg Zoo, an existing building on zoo grounds was modified to house several endangered species intended for a release program. The methods described above appear to be working very well for them, demonstrating the transferability of Omaha's Henry Doorly Zoo's techniques not only to other AZA-accredited zoos and aquariums, but to international facilities as well.

⁹ www.drsfostersmith.com/Product/Prod_Display.cfm?pcatid=3773&N=2004+113345

¹⁰ www.esuweb.com/cardfile.asp?ItemNumber=55112&IDProductRelationship=281

This chapter demonstrates that with a little imagination, institutions are able to follow the biosecurity recommendations handed down from the CBSG/WAZA Amphibian *Ex Situ* Conservation Planning Workshop with a relatively low investment of space and financial resources. Hopefully, this will motivate others to consider constructing their own IAR and attempt to save at least one species or one assemblage of amphibians.

REFERENCE

Zippel, K., R. Lacey, O. Byers (eds.) 2006. CBSG/WAZA Amphibian *Ex Situ* Conservation Planning Workshop Final Report. IUCN/SSC Conservation Breeding Specialist Group, Apple Valley, MN 55124, USA. Pp. 65.