

## 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 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; Woodhams et al., 2012). 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., tadpoles and recent metamorphs). 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 treatment “failures” result in Bd positive animals after a complete treatment course, it is important to evaluate the treatment protocol for any breakdown in clean handling and disinfection techniques that might have led to reinfection during or following treatments. Poor drug efficacy may be blamed inappropriately when Bd contamination sources are not identified in the treatment protocols.
- Very little data exists on infection burden (numbers of Bd zoospores present on an animal) as it relates to treatment efficacy. Some drugs may work for animals with low infection burdens, while the same drug may not be effective in clearing more heavily infected animals. Different concentrations of the same drug or use of a different drug or combination of drugs may be required at varying infection intensity.
- 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.
- While it is not recommended, 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 dessication (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.

### Itraconazole Treatment

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; Tamukai et al., 2011). 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 appears 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.

Potential 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 0.005 - 0.01% itraconazole protocols. There have been anecdotal reports of treatment-associated anorexia, corneal ulcers, kidney disease and deaths in some species (especially ranid frogs). When working with 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<sup>®</sup>

Oral Solution; Itrafungol<sup>®</sup> Oral Solution) to make a 0.005% or 0.01% treatment solution by dilution in amphibian Ringer's solution. The authors of this manual feel enough evidence and personal experience exists regarding treatment safety and efficacy to recommend primary usage of the 0.005% solution for the majority of cases. It may be that certain species or higher infection intensities may require use of the 0.01% concentration.

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<sup>®</sup>"), inexpensive plastic animal enclosures ("Pet Pals<sup>®</sup>"), glass aquariums, and large plastic storage containers ("Rubbermaid<sup>®</sup>").
  - 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 species of unknown risk 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<sup>®</sup> Oral Solution; Itrafungol<sup>®</sup> Oral Solution) is diluted in amphibian Ringer's solution (see Section 8.5 for recipe) to make a 0.005% treatment solution. Do not use this treatment solution on tadpoles (lower concentrations of itraconazole may be safe, see below).
  - To make each 100 ml of treatment solution, add 0.5 ml of 10mg/ml itraconazole to 99.5 ml of amphibian Ringer's solution (to make 1 liter of treatment solution add 5 ml of 10mg/ml itraconazole to 995 ml of amphibian Ringer's). This is equivalent to 50mg of itraconazole per liter of treatment solution. (To create a 0.01% solution, add 1ml of 10mg/ml itraconazole to 99ml Ringer's, or 10ml of 10mg/ml itraconazole to 990ml of Ringer's).
  - A recent case report documents successful use of 0.005% solution in naturally infected Wyoming toads (*Anaxyrus baxteri*) and White's tree frogs (*Litoria caerulea*) (Jones, et al., 2012).

- 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 or an alternate treatment strategy 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.
  - Protocols vary as to whether animals are rinsed (with clean filtered water or fresh amphibian Ringer’s solution) following the itraconazole bath. Prolonged exposure without rinsing may provide additional benefit of increased contact time of the drug with Bd zoospores, while it may also prolong negative effects such as skin irritation or adverse reactions in sensitive species.
  - After each daily treatment animals are returned to a clean and previously disinfected enclosure (see # 1 above).
5. 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 disinfect treatment containers after daily treatments, or use of treatment containers that can harbor waste water in crevices even after cleaning (such as curled edges on disposable plastic cups).
  - Failure to adhere to good biosecurity practices, such as touching “clean”, or freshly treated frogs after touching “dirty”, Bd contaminated waste water, supplies, or animals.
  - Failure to exercise strict quarantine and isolation procedures during treatment and post-treatment testing cycles; e.g., introduction of new untreated animals in the same room as treated animals.
  - 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.
6. 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.

### Chloramphenicol Treatment

The antibiotic chloramphenicol, typically used as an antibacterial agent, has been shown to be active against the Bd fungus. It has been reported to clear subclinical Bd-infected *Litoria caerulea* and was used successfully as part of a multi-modal treatment protocol in a small

number of terminally ill Bd-infected *L. caerulea* (Young et al., 2012). Three moribund *L. caerulea* were cleared of large infection burdens (>10,000 zoospore equivalents) using chloramphenicol and increased ambient temperature, while isotonic fluids were administered to address dehydration and electrolyte derangements.

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.
- Application by continuous immersion is very convenient for treatment of totally aquatic amphibian species and tadpoles.
- Treatment is inexpensive compared to itraconazole.
- Antibacterial action of chloramphenicol may provide an additional spectrum of protection against secondary bacterial infections.

Potential disadvantages to the use of chloramphenicol:

- The reported treatment protocol requires 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.
- Treatment protocol potentially requires daily handling of animals for up to 28 days, which may be stressful for some species. Practicality of feeding some species in a bath environment for that long also deserves consideration.
- Chloramphenicol is 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.

#### CHLORAMPHENICOL TREATMENT PROTOCOL

- The treatment solution is made from reagent grade chloramphenicol (chloramphenicol C0378; Sigma-Aldrich, St. Louis, MO). A stock solution is made by adding 200 mg of 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 a shallow bath of treatment solution for 2–4 weeks and must have constant exposure to the treatment solution. The treatment solution is changed daily.
- At the conclusion of treatment, PCR testing as described below 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 et al., 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-inoculation (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.

- **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. Voriconazole has shown potential as a treatment option. One study (Martel et al., 2011) cleared low-density infection burdens (17.8 +/- 15 Bd genomic equivalents) from midwife toadlets (*Alytes cisternasii*) using topical spray of a 1.25 µg/ml voriconazole

once daily for seven days. Of particular interest in this study was the finding of no toxic effect to *A. muletensis* tadpoles continuously exposed to voriconazole in water up to 12.5 µg/ml for seven days. While effectiveness of voriconazole in clearing Bd from tadpoles was not investigated in the study, it does imply a possibly safe treatment method for tadpoles.

- **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. Further research for use of this drug is needed. Protocols are readily found on line, though there has been little in the way of controlled studies to confirm efficacy or safety of this treatment and in most anecdotal cases, Bd has not been confirmed in animals prior to treatment. In one study (Bowerman et al., 2010), Bd PCR positive juvenile bullfrogs, *Lithobates catesbeiana*, were cleared of infection using bath solutions of 0.01% terbinafine and 1% ethanol for 5 minutes daily for 5 consecutive days. Four other anuran species and one caudate species were similarly cleared of infection using 0.005% terbinafine and 0.5% ethanol. All animals included appear to have been subclinical carriers; infection intensities in study subjects were not reported. One major limitation to this study is the failure to include treatment controls for ethanol alone.
- **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. 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.
- **Trimethoprim-sulfadiazine.** This combination appeared to have some fungistatic activity in a limited trial with dendrobatid frogs (Nichols et al., 2000).

### **Treatment of Animals Clinically Ill with Chytridiomycosis**

For animals that are clinically ill with chytridiomycosis (e.g., excessive skin shedding, lethargy, anorexia, poor righting reflexes, hunched posture, dermal hemorrhage; see Fig. 8.1), prognosis for survival during treatment is poor without further supportive treatment with supplemental electrolytes and antibiotics.

- 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.
- Subcutaneous administration of injectable isotonic electrolyte solution was administered to terminally ill frogs in an experimental study including chloramphenicol treatment (Young 2012).
- 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 and the solution is changed daily.
- Sodium chloride alone at concentrations greater than 3 ppt, reduced growth and motility of Bd. Concentrations from 1 – 4 ppt reduced infection loads and 3 – 4 ppt increased survival rates of Peron's tree frogs (*Litoria peronii*) experimentally infected with Bd (Stockwell 2012).
- 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.

### Notes On Treatment of Tadpoles

Many of the treatment options listed above are toxic to tadpoles at the same concentrations used for metamorphs. Few studies have been performed to specifically test safety and efficacy of treatments in tadpoles. Most references in the literature are anecdotal. Unless proven treatment techniques are available, consideration should be given and risk assessments performed to decide whether to allow metamorphosis prior to treatment of larvae.

- 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).
- Unpublished data (referenced in Young, et al. 2012) suggests that *Litoria ewingii* tadpoles showed no adverse effects after 3 – 4 weeks of continuous immersion in 20 mg/L chloramphenicol solution, the same concentration used to treat adult frogs.
- Additional unpublished data (referenced in Young et al. 2007) reports that very low concentrations of terbinafine (2 – 4 mg/L for 7 days) was non-toxic to tadpoles, though these concentrations are much lower than effective

treatment concentrations in adult frogs (50 – 100 mg/L; Bowerman et al., 2010).

- Elevated environmental temperature may prove useful as a treatment, adjunct, or control strategy for larvae in those species that can tolerate it. Bd-infected *Alytes obstetricans* tadpoles survived to metamorphosis, though were not cleared of infection, when raised at 21° C (Woodhams et al., 2011).

### Post-Treatment PCR Testing

Regardless of the treatment modality selected, animals should be tested to confirm successful clearance of Bd organisms whenever possible (See Sections 7.2–7.3). When PCR testing is not available, other diagnostic methods should be employed and animals should minimally be observed for signs of reinfection for at least 2 – 4 weeks following completion of treatment.

- Samples for PCR testing are obtained 10 – 14 days 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 to enhance sensitivity of detection. 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.

## 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. Refer to Supportive Care For Sick Amphibians.
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](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](http://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 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 between multiple examiners within a facility is most important.

This system for rhabditiform nematode larvae is used with direct wet-mount examination of feces through a 10x objective with standard light microscopy:

- 1+ = 1 or fewer larva identified for every 3 or more low powered fields
- 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**<sup>®</sup> 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<sup>®</sup>) 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 febendazole 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<sup>®</sup>). 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 without a micropipette. 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 SUPPORTIVE TREATMENTS

If any amphibian appears weak, lethargic, or debilitated, regardless of the cause, supportive treatments will usually be necessary, in addition to any primary treatments that may be directed toward specifically identified problems (such as chytrid or lungworm). Most important supportive treatments will include fluid and electrolyte replacement and antibiotic therapy to treat secondary bacterial infections. Amphibians that are stressed due to illness or any other physiological stressor may have depressed function of the skin electrolyte and immune defense systems. Any disruption of the skin barrier (especially as with chytridiomycosis) can lead to electrolyte loss and opportunistic invasion of environmental bacteria.

## 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 (CaCl<sub>2</sub>), 0.15 grams
  - Sodium bicarbonate (NaHCO<sub>3</sub>), 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 (MgSO<sub>4</sub>·7H<sub>2</sub>O), 8.6 grams
  - Calcium chloride (CaCl<sub>2</sub>), 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.

## Antibiotics

The most common types of bacteria present in the aquatic and semi-aquatic environment of amphibians are Gram-negative bacteria, such as *Aeromonas* spp., and these are the most common secondary invaders (Wright & Whitaker, 2001). Antibiotics should be selected with a strong spectrum of activity against Gram-negative bacteria.

- Enrofloxacin (Baytril) is a good first-choice antibiotic as it does have a good Gram-negative spectrum of activity and good distribution to tissues and bodily



fluids. The most commonly used form is a 2.27% (22.7 mg/ml) injectable solution (Baytril). This solution is very caustic to tissues, however, as it has a pH of approximately 10 and needs to be diluted prior to use.

- Dilute the 2.27% injectable Baytril 1:1 with 0.9% sodium chloride prior to use, to create 1.13% solution (11.35mg/ml). Dosage is .01mg enrofloxacin per gram of body weight. Therefore, calculate: (Body weight x .01) ÷ 11.35 = volume in ml. For animals weighing less than 10 grams, use micropipette for accurate dosing. If micropipette is not available, 1 drop of 1.13% solution from the tip of a 25 gauge needle is an adequate approximation. Continue once a day for at least 7 days. Ideally, antibiotic use should continue at least several days beyond the resolution of visible lesions. If lesions are not improving or become worse, consideration should be given to an alternate diagnosis or treatment.

## 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](http://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 cardiac activity has 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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