

Disease monitoring and biosecurity

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26.1 Introduction

Understanding and detecting diseases of amphibians has become vitally important in conservation and ecological studies in the twenty-first century. Disease is defined as the deviance from normal conditions in an organism. The etiologies (causes) of disease include infectious, toxic, traumatic, metabolic, and neoplastic agents. Thus, monitoring disease in nature can be complex. For amphibians, infectious, parasitic, and toxic etiologies have gained the most notoriety. Amphibian diseases have been linked to declining amphibian populations, are a constant threat to endangered species, and are frequently a hazard in captive breeding programs, translocations, and repatriations. For example, a group of viruses belonging to the genus *Ranavirus* and the fungus *Batrachochytrium dendrobatidis* are amphibian pathogens that are globally distributed and responsible for catastrophic population die-offs, with *B. dendrobatidis* causing known species extinctions (Daszak *et al.* 1999; Lips *et al.* 2006; Skerratt *et al.* 2007). Some infectious diseases of amphibians share similar pathological changes; thus, their detection, recognition, and correct diagnosis can be a challenge even by trained veterinary pathologists or experienced herpetologists.

This chapter will introduce readers to the most common amphibian diseases with an emphasis on those that are potentially or frequently lethal, and the techniques involved in disease monitoring. It will also outline methods of biosecurity to reduce the transmission of disease agents by humans. We start by covering infectious, parasitic, and toxic diseases. Next, surveillance methods are discussed, including methods for sample collection and techniques used in disease diagnosis. Finally, biosecurity issues for preventing disease transmission will be covered, and we provide protocols for disinfecting field equipment and footwear.

26.2 Amphibian diseases of concern

Amphibians are susceptible to a variety of pathogens, including internal and external parasites, viruses, bacteria, and fungi. Each of the three major life stages of amphibians (embryos, larvae, and adults) has a distinct suite of diseases, with some overlap between life stages. Aquatic amphibian embryos and larvae share many diseases with fish, whereas post-metamorphic stages often share few infectious diseases with earlier life stages. For detailed information on the amphibian diseases, we recommend that readers consult recent reviews (e.g. Converse and Green 2005a, 2005b; Green and Converse 2005a, 2005b) and the veterinary literature (e.g. Wright and Whitaker 2001).

26.2.1 Infectious diseases

Major infectious diseases for each amphibian life stage are summarized in Tables 26.1–26.3. Many viruses have been reported in amphibians, and include *Ranavirus*, herpesvirus, and adenovirus (Converse and Green 2005a, 2005b; Green and Converse 2005a, 2005b). Of these, *Ranavirus* has been the most significant contributor to population declines, resulting in significant morbidity and mass mortality (Daszak *et al.* 1999; Green *et al.* 2002; Cunningham *et al.* 2007). In North America, ranaviruses are responsible for the majority of catastrophic die-offs in ambystomid salamanders and late-stage anuran larvae, with the number of reported cases each year exceeding all other pathogens by three to four times (Green *et al.* 2002; Muths *et al.* 2006). Although many die-offs have been with common species, declines in several species of conservation concern (e.g. *Rana muscosa*, *Rana aurora*, *Bufo boreas*, and *Ambystoma tigrinum stebbinsi*) have been reported (Jancovich *et al.* 1997; Converse and Green 2005a). There is evidence that ranaviruses may function as a novel or endemic pathogen, with the former likely associated with the movement of infected amphibians by humans (Storfer *et al.* 2007). Anthropogenic stressors also may facilitate emergence (Forson and Storfer 2006; Gray *et al.* 2007a). Additionally, subclinically infected individuals (i.e. those that do not appear sick) may serve as reservoirs for more susceptible amphibian species (Brunner *et al.* 2004).

Likewise, numerous bacteria have been cultured from anurans (Mauel *et al.* 2002). Of these, *Mycobacterium liflandii*, a mycolactone-producing mycobacteria, is of concern because it is closely related to the human pathogen *Mycobacterium ulcerans* (Yip *et al.* 2007), which causes severe skin lesions in humans. Nevertheless, *Aeromonas hydrophila* remains the most recognized bacterial pathogen in amphibians because of its association with red-leg disease

Table 26.1 Significant diseases of amphibian eggs and embryos.

Disease agent	Common host species	Mortality rate	Organ of choice	Test methods
Lucke tumor herpesvirus	<i>Rana pipiens</i> only	0	Mesonephros or whole embryo	Culture on <i>Rana pipiens</i> cell line; PCR
<i>Chlamydomonas</i> (symbiotic alga) ¹	<i>Ambystoma</i> spp.	0	Egg capsule	Gross or microscopic exam
Watermolds ²	<i>Bufo</i> spp., <i>Hyla</i> spp., <i>Pseudacris</i> spp., <i>Rana</i> spp., <i>Ambystoma</i> spp., <i>Taricha</i> spp.	Variable	Egg capsule	Culture; histology; DNA sequencing
<i>Microsporidium schuetzi</i>	<i>Rana pipiens</i>	< 10%	Whole swollen eggs	Histology; electron microscopy
Tetrahymena/Glaucoma (ciliated protozoa)	<i>Ambystoma</i> spp.	15–25%	Egg capsule, brain and subcutis of embryos/larvae	Submerged exam of eggs/embryos under dissecting microscope; histology; exam by protozoologist

¹*Chlamydomonas* sp. is a symbiotic blue-green alga in the egg capsule of *Ambystoma maculatum* in eastern North America and *Ambystoma gracile* in western North America, and not considered a disease agent.

²Watermold infections (oomycetes of several genera) referred to as saprolegniasis.

(Green and Converse 2005a). However, it is important to note that red-leg disease is a gross descriptor of a specific lesion (i.e. swollen red legs) and not specific for a particular etiology. Many pathogens (e.g. *Ranavirus*, *A. hydrophila*, alveolates) can cause edema (i.e. swelling) and erythema (reddening) in amphibians (Figure 26.1a). This emphasizes the importance of diagnostic testing to determine the correct pathogen causing the disease.

Finally, numerous fungal and fungus-like organisms (Converse and Green 2005a, 2005b; Green and Converse 2005a, 2005b) and newly characterized pathogens (Davis *et al.* 2007) are known to cause catastrophic mortality of amphibian populations. *B. dendrobatidis* (Figure 26.1b) has resulted in global population declines and species extinctions (Wake and Vredenburg 2008). The newly discovered alveolate organism has only been diagnosed in a few

Table 26.2 Significant diseases of larval amphibians.

Disease agent	Common host species	Mortality rate	Organ of choice	Test methods
Ranaviruses	<i>Bufo</i> spp., <i>Hyla</i> spp., <i>Rana</i> spp., <i>Pseudacris</i> spp., <i>Ambystoma</i> spp., <i>Notophthalmus</i> spp.	50–99%	Liver, skin ulcers mesonephroi ¹	Culture at 20–25°C; PCR on liver, spleen, skin ulcers, mesonephroi
<i>Batrachochytrium dendrobatidis</i>	<i>Rana</i> spp., <i>Pseudacris</i> spp.	0%	Oral disc, toe tips	Histology; PCR; culture
Watermolds ²	<i>Bufo</i> spp., <i>Hyla</i> spp., <i>Pseudacris</i> spp., <i>Rana</i> spp., <i>Ambystoma</i> spp., <i>Taricha</i> spp.	Variable	Oral disc, skin	Culture; histology
<i>Ichthyophonus</i> sp.	<i>Pseudacris</i> spp., <i>Rana</i> spp., <i>Ambystoma</i> spp.	0–50%	Skeletal muscle	Histology
<i>Perkinsus</i> -like organism	<i>Rana</i> spp., rarely <i>Hyla</i> spp., rarely <i>Pseudacris</i> spp.	5–99%	Liver	Histology; PCR
Tetrahymena/glaucoma (ciliated protozoa)	<i>Ambystoma</i> spp.	15–25%	Egg capsule, brain and subcutis of embryos/larvae	Submerged exam of eggs/embryos under dissecting microscope; histology; examination by protozoologist
<i>Ribeiroia ondatrae</i>	<i>Bufo</i> spp., <i>Pseudacris</i> spp., <i>Rana</i> spp., <i>Ambystoma</i> spp.	Variable	Skin around vent and proximal hindlimbs	Examination by parasitologist; PCR
Other metacercariae	Most aquatic genera	Low	Parasite	Examination by parasitologist
<i>Lernaea</i> sp. (“anchorworm”)	<i>Rana</i> spp.	Low	Parasite in skin	Examination by parasitologist
Leeches	<i>Rana</i> spp.	Low	Parasite	Examination by parasitologist

¹Mesonephroi, “body kidneys” (versus pronephroi or “head kidneys” found only in larvae); “true” kidneys of reptiles, birds, and mammals are metanephroi.

²Watermold infections (oomycetes of several genera) referred to as saprolegniasis.

Table 26.3 Significant diseases of post-metamorphic amphibians.

Disease agent	Common host species	Mortality rate	Organ of choice	Test methods
Ranaviruses	<i>Bufo</i> spp., <i>Hyla</i> spp., <i>Pseudacris</i> spp., <i>Rana</i> spp., <i>Ambystoma</i> spp., <i>Notophthalmus</i> spp.	Low in adults; variable in recently metamorphosed amphibians	Liver, skin ulcers mesonephroi	Culture at 20–25°C; PCR on liver, spleen, skin ulcers, mesonephroi; electron microscopy
Lucke tumor herpesvirus	<i>Rana pipiens</i>	Variable in >2 yr old <i>Rana pipiens</i> only	Mesonephroi	Histology of tumors
<i>Batrachochytrium dendrobatidis</i>	Most anuran genera	Very high in many anurans especially at high elevations in tropical latitudes	Skin of pelvic patch, toe webs	Histology; PCR; culture; electron microscopy
<i>Ichthyophonus</i> sp.	<i>Rana</i> spp., <i>Ambystoma</i> spp., <i>Notophthalmus</i> spp.	Very low	Skeletal muscle	Histology
<i>Amphibiothecum penneri</i> ¹	<i>Bufo</i> spp.	0	Ventral skin nodules	Cytology of discharge; histology of nodule
Hepatoozoon spp.	<i>Rana</i> spp.	Low	Blood smear; liver	Cytology; histology
<i>Ribeiroia ondatrae</i>	<i>Bufo</i> spp., <i>Pseudacris</i> spp., <i>Rana</i> spp., <i>Ambystoma</i> spp.	0	Skin around vent, proximal hindlimbs and at tip of urostyle	Examination of metacercaria by a parasitologist; PCR; radiographs of malformations
<i>Rhabdias</i> spp. (nematode lungworm)	<i>Bufo</i> spp. <i>Rana</i> spp.	Unknown	Lungs	Visible at dissection; histology; examination by a parasitologist

¹*Amphibiothecum* (formerly *Demosporidium*) *penneri*, referred to as dermosporidiosis.

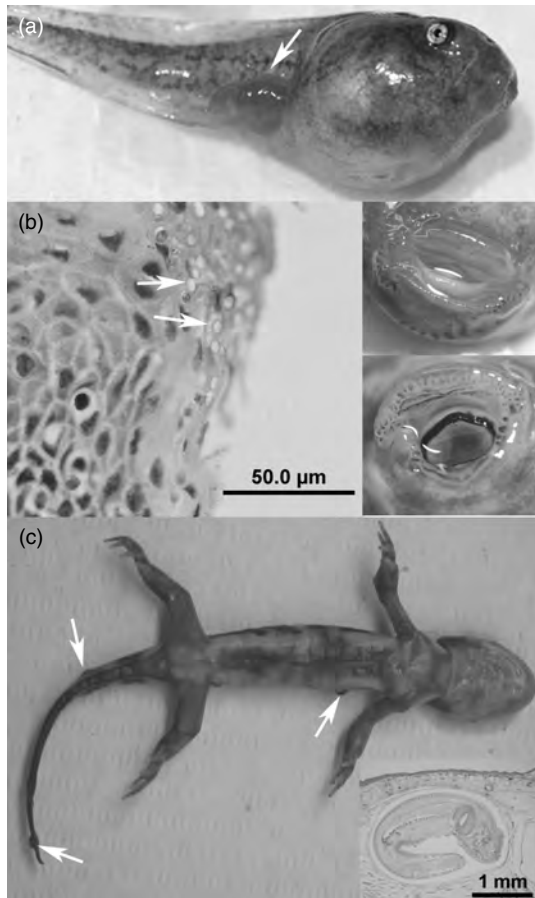


Fig. 26.1 (a) Tadpoles with swollen bodies and swollen red legs (arrow) are often diagnosed as red-leg disease but the etiology is varied and may include *Aeromonas hydrophila*, *Ranavirus*, and alveolates. (b) The amphibian fungus, *Batrachochytrium dendrobatidis* (arrows), infects the keratin-producing cells of amphibians. Tadpole skin is not keratinized; rather, only their 'teeth' contain keratin. Grossly, this is seen by loss of pigmentation (upper inset) of the tooth rows. Lower inset is of a normal tadpole for comparison. (c) Trematode cercaria encyst within the skin (arrows) and body cavities of amphibians serving as a secondary host and may be easily seen grossly. Histologically, the organisms are found in thin-walled cysts (inset).

isolated geographical areas so far (Davis *et al.* 2007). Still other organisms, such as the water molds *Saprolegnia*, may be beneficial (e.g. by facilitating decomposition of dead eggs) but also have the potential to be opportunistic pathogens of amphibians at any life stage (Converse and Green 2005a, 2005b; Green and Converse 2005a, 2005b).

26.2.2 Parasitic diseases

As with any species, parasites are commonly found on and in amphibians (Figure 26.1c). External parasites include leaches, anchorworms, and mites, whereas internal parasites include various trematodes, cestodes, nematodes, and protozoa (Converse and Green 2005a, 2005b; Green and Converse 2005b; Wright and Whitaker 2001). Many species of helminthes (trematodes, cestodes, nematodes) have been documented in amphibians, and often they are considered incidental (Miller *et al.* 2004), but their presence may be an indicator of stress or aquatic food-web restructuring related to human land use (Johnson and Lunde 2005; Gray *et al.* 2007b). Likewise, many protozoans (e.g. myxozoa) are often considered incidental findings but their numbers may increase when amphibians are stressed, and they may potentially contribute to morbidity.

26.2.3 Toxins

Contaminants in the environment may kill larvae or post-metamorphs (Relyea 2005, 2009), and may have non-lethal impacts including reducing growth, impacting metamorphosis, disrupting gonadal development and secondary sex characteristics, or causing musculoskeletal, skin, and visceral malformations (Boone and Bridges 2003; Davidson *et al.* 2007; Ouellet *et al.* 1997; Storrs and Semlitsch 2008). Often these changes are not detected by external examinations until metamorphosis is complete or until the animals attain a size for reproduction. Amphibians are often considered sentinels or bio-indicators of environmental quality because they are sensitive to toxins and many species have the potential to be exposed to stressors in aquatic and terrestrial systems due to their typical biphasic life cycle (Blaustein and Wake 1995).

26.3 Disease monitoring: detection and diagnosis

26.3.1 Disease surveillance

Recently, the World Animal Health Organization (the OIE; www.oie.int/eng/en_index.htm) included two amphibian diseases (chytridiomycosis and ranaviral disease) on their listing of reportable diseases. The OIE listing provides the impetus for disease surveillance and required testing of amphibians prior to transport among states or between nations. The need for required testing of amphibians for pathogens has been expressed by several researchers (Gray *et al.* 2007a; Griffiths and Pavajeau 2008; Picco and Collins 2008). Historically, pre-transport pathogen testing and health certification for amphibians has

been essentially non-existent, unlike for domestic livestock and pets and some wild mammals (e.g. cervids). The OIE has established guidelines for surveillance and requirements necessary for countries to declare *Ranavirus*-free status (www.oie.int/eng/norms/fcode/en_chapitre_2.4.2.htm#rubrique_ranavirus) and *B. dendrobatidis*-free status (www.oie.int/eng/normes/fcode/en_chapitre_2.4.1.htm#rubrique_batrachochytrium_dendrobatidis). The OIE-approved methods for conducting surveillance and diagnosis of infection are in development. In the meantime, guidelines from the 2006 OIE Manual of Diagnostic Tests for Aquatic Animals (www.oie.int/eng/normes/fmanual/A_summry.htm) and from the fisheries industry (USFWS and AFS-FHS 2005) can be helpful for general monitoring of amphibian population health. In general, the criteria of a population health assessment should include (1) determination of the status and trends of amphibian pathogens, (2) determination of the risk of disease for threatened or endangered amphibians, (3) investigation of unexplained population declines, (4) evaluation of populations following a morbidity or mortality event, (5) detection of pathogens in non-indigenous species, (6) evaluation of a site or population prior to translocation, (7) evaluation of sympatric amphibians prior to release of captive-raised animals, and (8) the potential for amphibians and their diseases to “piggy back” with fish translocation.

Disease testing should not focus on one pathogen. For surveillance programs, we recommend that animals are tested for infection by at least the OIE pathogens: ranaviruses and *B. dendrobatidis*. For diagnosis of morbid or dead individuals, we encourage a full diagnostic work-up (i.e. necropsy, histology, bacterial culture, virus testing, and parasite testing) to attempt to identify all etiologic agents. It is important to note that simultaneous infection by multiple pathogens is possible. Further, histological examination of organs often is required to determine which of the pathogens identified are causing the changes responsible for the diseased state (Miller *et al.* 2008, 2009). Histological examination is also important in discovering introduced pathogens or pathogens that have not been described previously (Longcore *et al.* 1999; Davis *et al.* 2007).

Population health assessments can include non-lethal or lethal collection of tissue samples from individual amphibians (Greer and Collins 2007), and collection of environmental samples (e.g. water, soil; Walker *et al.* 2007). Ideally, we recommend that tissue samples are collected from all species in a community and from pre- and post-metamorphic life stages. Amphibian species differ in susceptibility to pathogens, and some age classes may serve as a reservoir (e.g. larval for *B. dendrobatidis* and adults for *Ranavirus*; Daszak *et al.* 1999; Brunner *et al.* 2004; Schock *et al.* 2008). Further, some infectious diseases become evident only after the post-metamorph has overwintered (e.g. Lucke tumor herpesvirus, *Amphibiothecum* (formerly *Dermosporidium penneri*). The lack of gross signs

of disease also does not imply healthy populations. We and others have found tadpoles with no signs of illness but that are infected with ranaviruses (Gray *et al.* 2007a; Harp and Petranka 2006; Miller *et al.* 2009). Laboratory studies have demonstrated that amphibian pathogen infection and mortality rates frequently track each other (e.g. Brunner *et al.* 2007); thus, high prevalence in a population could signal that a die-off is imminent.

In some cases, it may not be possible to collect sufficient tissue for disease testing. For example, small amphibians (e.g. *Bufo* larvae) may not have adequate tissue for tests, especially for toxicological analysis. Also, non-lethal testing may be required because a species is listed as a conservation concern. We found that testing for *Ranavirus* from tail clips results in about 20% false-negatives (D. L. Miller and M. J. Gray, unpublished results). In cases when a small amount of tissue is collected, multiple individuals within a species could be pooled to acquire sufficient tissue for testing. If contaminants are suspected as the cause of a die-off, we also recommend collecting and testing water and sediment at the amphibian breeding site.

Monitoring for malformations can be challenging, because typically malformed individuals have low survival. Although amphibian malformations have been documented for many years (Rostand, 1958), an increase in malformation rates occurred in the late twentieth century (Johnson and Lunde 2005). Generally, malformation studies have targeted recently metamorphosed amphibians (Meteyer *et al.* 2000), because metamorphs with prominent abnormalities are quickly removed from the population by predation or starvation. Additionally, the bony skeleton of metamorphosed amphibians is more conducive for radiographically visualizing deformities compared to the cartilaginous skeleton of larvae. However, monitoring of larval abnormalities is needed because it is likely that some abnormalities prevent metamorphosis, thus are not detected in post-metamorphic cohorts.

Finally, comprehensive disease surveillance should include captive amphibians in zoological and ranaculture facilities, because disease transmission can occur between captive and free-ranging populations. Maintenance of health in zoological facilities is especially important for rare species or in captive breeding populations intended for release. High densities in ranaculture facilities, pet shops, and stores that sell amphibians (e.g. *Ambystoma tigrinum*) for fishing bait can be cauldrons for disease transmission and pathogen evolution (Picco and Collins 2008). Ranaviruses isolated from ranaculture facilities and bait shops appear to be more virulent than wild strains (Majji *et al.* 2006; Storfer *et al.* 2007). This emphasizes the importance for disease monitoring at facilities with captive amphibians. In the event of a die-off in a captive facility, freshly dead animals should be submitted for diagnostic evaluation. Live

animals that are infected should be euthanized or treated if a treatment exists (discussed in section 26.4.3), and facilities decontaminated with bleach or an equivalent disinfectant (discussed in section 26.4.2).

26.3.2 Sample size

Determination of statistically appropriate sample sizes for amphibian disease surveillance remains in its infancy. Although not established for amphibians, health assessment of fish is based on the minimum assumed pathogen prevalence level (APPL). The commonly used APPLs in aquatic health investigations are 2, 5, and 10% (Lavilla-Pitogo and de la Pena 2004; USFWS and AFS-FHS 2005). The APPL is used with an estimate of amphibian population size to determine the number of individuals that should be tested to have 95% confidence in pathogen detection. If it is assumed that APPL is 10%, required sample size ranges from 20 to 30 depending on the size of the amphibian population (Table 26.4). Required sample size increases with decreasing APPL (Table 26.4). Unpublished findings of the US Geological Survey National Wildlife Health Center suggest that APPL for ranaviruses, *B. dendrobatidis*, and alveolates is 10% or less (Table 26.5). In Tennessee, USA, health monitoring of two common anuran species inhabiting farm ponds revealed 29% prevalence for *Ranavirus*, 0% for *B. dendrobatidis* and alveolates, and 43% for parasites (Miller *et al.*, 2009). *Ranavirus* prevalence in plethodontid salamanders in the southern Appalachian Mountains can range from 3 to 81% depending on the watershed (M. J. Gray and D. L. Miller, unpublished results). Thus, we recommend that biologists determine required sample sizes for amphibian disease monitoring using either 5 or 10% APPL (Table 26.4).

26.3.3 Sample collection and shipment

Sample collection may include whole live animals, dead animals, sections of tissues, swabs of lesions or orifices, environmental samples, or sympatric species. It is important to wear disposable gloves when handling amphibians and to change gloves between animals. This is necessary to prevent disease transmission between amphibians and to protect biologists from zoonotic diseases (discussed in section 26.4). Gutleb *et al.* (2001) and Cashins *et al.* (2008) reported that disposable gloves (especially latex gloves) may be toxic to amphibian larvae. Therefore, when handling amphibians, biologists and researchers should use disposable vinyl gloves that have been rinsed with distilled or sterilized water (Cashins *et al.* 2008).

Mortality events involving all amphibian species should be investigated, even if it is not part of a disease surveillance program. There is a paucity of information

Table 26.4 Sample size (i.e. number of amphibians) to assure 95% confidence in detection of pathogens in a population (modified from USFWS and AFS-FHS 2005).

Estimated population size	Number of amphibians for	
	5% APPL	10% APPL
100	45	23
500	55	26
2000	60	27
>10 000	60	30

APPL, assumed pathogen prevalence level.

Table 26.5 Previously unreported low disease prevalence in free-ranging amphibian populations in the USA (US Geological Survey National Wildlife Health Center).

Pathogen	Host species	Life stage	US state	Sample size	Prevalence (number positive)	Test method	Case number
<i>Ranavirus</i>	<i>R. catesbeiana</i>	L	OR	15	7% (1)	Culture	44276
	<i>Pseudacris maculata</i>	L	WY	11	18% (2)	Histology	4779
Perkinsus-like organism	<i>R. catesbeiana</i>	L	OR	12	8% (1)	Histology	44276
	<i>R. sphenoccephala</i>	L	FL	15	27% (4)	Histology	4864
	<i>R. sphenoccephala</i>	L	LA	12	8% (1)	Histology	18626
	<i>R. sphenoccephala</i>	RM	MD	14	21% (3)	Histology	18761
	<i>R. sphenoccephala</i>	L	MS	11	9% (1)	Histology	18642
<i>Ichthyophonus</i>	<i>R. sphenoccephala</i>	L	FL	19	11% (2)	Histology	4864
	<i>R. grylio</i>	L	FL	16	19% (3)	Histology	4864
	<i>R. sphenoccephala</i>	L	LA	12	8% (1)	Histology	18626
	<i>R. clamitans</i>	L	ME	16	31% (5)	Histology	4824

L, larvae; RM, recently metamorphosed.

on the occurrence of pathogen-related die-offs in amphibian populations. The majority of samples submitted to diagnostic laboratories are from biologists that encountered a dead or morbid amphibian during other work activities. Morbid or freshly dead amphibians are preferred, because amphibians decompose rapidly. Decomposed carcasses are not suitable for cultures, histology, and parasitological examinations, but may have limited diagnostic usefulness for molecular tests that detect pathogens and for toxicological analyses. In general, we recommend that amphibians be collected live or within 24 h of death. Mummified

(i.e. desiccated) carcasses with dry, leathery, and stiff digits or limbs usually have limited diagnostic usefulness.

Dead amphibians should be collected, put individually in plastic bags (e.g. Nasco Whirl-Pak® bags), and placed on ice for transport. Live amphibians can be placed in separate plastic containers and humanely euthanized (Baer 2006) via transdermal exposure for 10 min to tricaine methanesulfonate (100–250 mg/L) or benzocaine hydrochloride (> 250 mg/L or 20% benzocaine over-the-counter gel; Oragel, Del Pharmaceuticals, Uniondale, New York, USA) after returning from the field. It is important that amphibians are bagged separately to prevent cross-contamination of samples. Biologists that are experienced in blood collection may collect blood antemortem from the ventral vein in adult anurans or tail vein in salamanders, or collect blood antemortem or immediately postmortem from the heart of larvae or adults (Wright and Whitaker 2001). Blood can be tested for various biochemical parameters and examined for cellular composition, blood parasites, and viral inclusions (discussed in section 26.3.4).

We recommend that half of the individuals collected are frozen immediately for cultures and molecular tests. Samples can be frozen in a standard -20°C freezer if stored for short duration (< 1 month); otherwise, samples should be stored in a -80°C freezer. The other half of samples should be promptly fixed in 75% ethanol or 10% neutral buffered formalin for histology. For the first 2–4 days of fixation, the volume of fixative should be 10 times the volume of the animals. After this initial fixation, carcasses can be stored in a smaller volume of fixative that is sufficient to cover the tissues. The body cavity of amphibians that are more than 1 g in body mass should be cut along the ventral midline prior to immersion in fixative to assure rapid fixation of internal organs. Body cavities of frozen individuals should not be opened.

Special processing is required for amphibians with skin, digital, limb, head, or vertebral abnormalities. Whenever possible, amphibians with suspected malformations should be submitted alive for examinations. Dead individuals should be promptly frozen until time exists to properly fix individuals. Fixation can be done with ethanol or formalin but should be done in a pan so that carcasses can be positioned on a flat surface with limbs and digits extended from the body during fixation. Positioning amphibians in the standard museum configuration is ideal. Digits and limbs may be taped in position prior to fixation. Amphibians should be covered with fixative and additional fixative added if a significant amount evaporates. Placing a cover over the pan will help reduce evaporation. After 2–4 days of fixation, the carcass and limbs will be hardened in position and may be stored in a smaller volume of fixative. The

positioning described above is necessary for radiographic examination of the malformation.

Given that it often is not possible to obtain collection permits for threatened amphibians, alternative sampling may be necessary. Two alternatives are (1) capture–release studies and (2) collection of “sentinel” sympatric amphibian species. Capture–release studies can be used to collect swabs of external tissues, blood, or fecal samples. Swabs appear to be a reliable technique to test for *B. dendrobatidis* (Kriger *et al.* 2006); however for ranaviruses, false-positive and -negative test results are greater than for tail clips and both of these non-lethal techniques have more false results than testing internal organs (D. L. Miller and M. J. Gray, unpublished results). Swabs are typically performed in the oral then cloacal regions, and the swab stored in its packaging container or a microcentrifuge tube. Swabs should be put on ice and frozen similar to tissues. An accepted protocol for swabbing amphibians for *B. dendrobatidis* testing using PCR (discussed in section 26.3.4) has been reported by Brem *et al.* (2007) and can be found at www.amphibianark.org/chytrid.htm. Briefly, the amphibian should be gently but firmly swabbed in a sweeping motion five times at each of the following five locations (for a total of 25 times): rear feet (toe webbing), inner thighs, and ventral abdomen. Occasionally, modifications to this technique are necessary for salamanders (Brem *et al.* 2007). Swabs for *B. dendrobatidis* testing by PCR may be stored in 70% ethanol. Collecting common sympatric species for health assessment can provide insight into the presence of amphibian pathogens at a site, but does not allow for direct health assessment of the species of concern, which may differ in susceptibility.

Shipment of live, freshly dead, or frozen specimens must be via an overnight courier and according to the specific courier guidelines. For fixed specimens, overnight shipment is unnecessary. General guidelines for shipment include triple packaging and labeling each layer of packaging with a waterproof writing utensil. Commonly, the first package layer is a specimen in a Whirl-Pak® bag. The second layer is a larger sealable plastic bag in which multiple specimens are placed. If the first package layer contains liquid (e.g. ethanol), paper towel should be added to the second package to absorb any liquid if a spill occurs. The third package typically is a padded box or shipping cooler. For frozen specimens, adequate ice packs or dry ice should be added around the secondary package. It is vital that the package contains a detailed list of all contents, a description of requested services, and the contact information of the shipper. The tracking information should be provided to the recipient prior to package arrival.

26.3.4 Diagnostics

Several tools are available for diagnosing amphibian diseases but generally require some level of specialized expertise to perform. Commonly used diagnostic tools include necropsy, histology, cytology, bacterial culture, virus isolation, fecal floatation, electron microscopy, molecular modalities, and radiology. Most of these tests can be performed on samples collected from dead or live amphibians. Fresh or frozen tissues can be used for most tests, and are necessary for virus isolation. Frozen tissues are not appropriate for histology or cytology; rather, preserved tissues are used. Although formalin-fixed specimens are preferred for histological examination, ethanol-fixed specimens may also be used. Blood can be used for cell counts to assess immune function and to look for inclusion bodies that can be diagnostic for certain pathogens. Blood also may be tested for the presence of antibody response to various diseases. Examples of laboratories that currently test for amphibian diseases in Australia, New Zealand, Europe, and the USA include Australian Animal Health Laboratories (AAHL), Geelong, Victoria, Australia (www.csiro.au/places/aahl.html), Gribbles Veterinary Pathology, Australia and New Zealand (www.gribblesvets.com/), Exomed, Berlin, Germany (www.exomed.de/), Hohenheim University (R. Marschang), Stuttgart, Germany, Wildlife Epidemiology, Zoological Society of London (ZSL), London, UK, The University of Georgia Veterinary Diagnostic and Investigational Laboratory, Tifton, GA, USA (www.vet.uga.edu/dlab/tifton/index.php), University of Florida (J. Wellehan), College of Veterinary Medicine, Gainesville, FL, USA, and National Wildlife Health Center, Madison, WI, USA (www.nwhc.usgs.gov/).

There are advantages and disadvantages to the various tests available (Table 26.6). Necropsy allows for identification and documentation of external and internal gross changes. Histological and cytological examination allows for identification of changes at the cellular level and is generally necessary to document disease versus infection. Virus isolation is the process of culturing a virus which is necessary to determine the presence of live virus and to perform some molecular tests used in identifying viral species (e.g. sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS/PAGE) and restriction fragment length polymorphism (RFLP)). One caveat is that some viruses are difficult to culture, thus infection cannot be ruled out based solely on negative isolation results. Electron microscopy is used for identifying key features of parasites or other infectious agents (e.g. *B. dendrobatidis*, *Ranavirus*, herpesvirus), documenting intracellular changes or changes to the cellular surface, and confirmation of cultured virus. Electron microscopy can be performed on fresh, fixed, or paraffin-embedded tissues. Radiology allows

Table 26.6 Advantage and disadvantages of diagnostics tests for amphibian pathogens given the type of sample.

Sample type	Tests	Pathogen	Advantages	Disadvantages
Live animal	Necropsy, histology, cytology, hematology, virus isolation, bacterial culture, toxicological analysis, parasitology, PCR	Viruses, bacteria, fungi, parasites, toxins	Observe behavior, least chance of contamination, blood collection is possible	Difficulty in transport, stressful to animal
Fresh tissue (including whole dead organisms)	Necropsy if whole animal, histology, cytology, virus isolation, bacterial culture, toxicological analysis, parasitology, PCR	Viruses, bacteria, fungi, parasites, toxins	Can isolate live pathogens	If advanced postmortem autolysis, then of limited value
Frozen tissue	Virus isolation, bacterial culture, PCR	Viruses, bacteria, fungi, parasites, toxins	Can isolate live pathogens	Limited value for histology (freeze artifact)
Swab	Virus isolation, bacterial culture, PCR	Viruses, bacteria, fungi	Non-lethal, may detect shedders	False positives and negatives are possible
Fixed tissue	Histology, PCR	Parasites, bacteria, fungi, viruses,	Can see cellular changes due to disease	Cannot isolate live pathogens

for documentation of bone structure or the presence of foreign bodies, including certain parasites (e.g. *Ribeiroia metacercariae*).

Molecular testing is becoming increasingly popular and affordable for disease diagnostics. It is especially useful for endangered species, as non-lethal sampling can yield accurate results. Specifically, it can be performed on fresh, fixed or paraffin-embedded tissues, swabs, blood, and feces. For testing via PCR, one caveat is that a positive PCR result only confirms the presence of the pathogen whether it is dead or alive. Thus, it is important to perform supportive tests (e.g. virus isolation, histological examination) to differentiate between infection and disease. Either conventional or real-time PCR (qPCR) may be used, depending on the availability of known primer sequences and the purpose of the test. For quantifying viral presence and infection (Yuan *et al.* 2006; Storfer *et al.* 2007), qPCR is most ideal (Brunner *et al.* 2005; Pallister *et al.* 2007). However, if

sequencing is desired, which is often necessary to identify the species of a pathogen, conventional PCR is necessary.

There are three standard methods for characterizing amphibian malformations: (1) dissection, (2) radiography, and (3) clearing and staining. Dissection of a carcass is tedious, as it usually requires careful removal of muscles from limbs, head, and axial skeleton. Dermestid beetles (*Dermestes maculatus*) might be used to remove muscles and soft tissues from an amphibian carcass, but re-assembly of bones of vertebrae, limbs and digits can be very challenging and time-consuming. Radiography is the preferred diagnostic method for investigating and documenting abnormalities of the skeleton (Meteyer *et al.* 2000). A major limitation of radiography is that cartilage is invisible; hence, detection of abnormalities of cartilage is not possible. Instead, the clearing-and-staining method commonly used in teratological studies of embryos is recommended when both cartilage and bone need to be examined (Kimmel and Trammell 1981; Schotthoefer *et al.* 2003). This method involves “clearing” the skin, muscles, and viscera by immersion in potassium hydroxide. The bones are stained red with Alizarin Red, and cartilage is stained blue using Alcian Blue stain. Clearing and staining is the preferred method to evaluate larval amphibian skeletal abnormalities.

Regardless of the diagnostic tests employed, interpretation of the test results must be done with caution and knowledge of the amphibian pathogen that is being tested (Table 26.6). The type of sample must be considered when targeting a pathogen. For example, infection of *Ranavirus* is best diagnosed from internal organs otherwise environmental contamination (e.g. water or soil) cannot be ruled out. Nonetheless, documentation of ranaviruses from skin surfaces does provide evidence of environmental exposure. In contrast, *B. dendrobatidis* is commonly tested from skin surfaces in adults or mouth parts in larvae, because this pathogen infects only keratinized tissue (Kriger *et al.* 2006; Skerratt *et al.* 2008). However, histology is generally required to distinguish between *B. dendrobatidis* exposure and infection when gross lesions are not observed. In contrast, some pathogens (e.g. alveolates, *Ichthyophonus* spp., *Ribeiroia ondatrae*) may not be identifiable from external swab preparations and often specialized techniques are required (e.g. clearing or radiography for *R. ondatrae*). In addition, one must keep in mind that there is a difference between malformations and deformities. Malformations are those abnormalities that arise during growth and development (organogenesis) in which the organ or structure fails to form normally. A deformity is an abnormality that naturally occurs to a normal organ or structure, such as an

amputation or wound. It is often difficult to determine, even in radiographs, whether an abnormality is a malformation or a deformity.

26.4 Biosecurity: preventing disease transmission

Lethal infectious diseases of amphibians may be endemic and emerge in response to stressors, whether anthropogenic or natural (Carey *et al.* 2003). Disease emergence also may occur through geographical transport of pathogens (Jancovich *et al.* 2005; Storfer *et al.* 2007). Ranaviruses, *B. dendrobatidis*, the alveolate organism, and *Ichthyophonus* spp. are well established in many regions of the world; however, it is likely that some amphibian species have never been exposed to these agents. Further, in areas with multiple endemic pathogen strains or species (e.g. ranaviruses), slight variations in genetic coding can increase virulence (Williams *et al.* 2005). Thus, an endemic strain may function as a novel pathogen to amphibian populations outside the region where the pathogen evolved. This may be especially true with amphibian pathogens given the limited mobility of their host. Hence, prevention of the spread of endemic diseases to naïve populations or species remains a high conservation priority. Health examinations of amphibian populations and good biosecurity methods need to be employed because often little is known about the life cycles of infectious diseases, modes of transmission, and the persistence of the pathogen within and outside the amphibian host.

Preventing mechanical transmission of pathogens and contaminants from one location to another by equipment, supplies and people is the purpose of biosecurity. Biosecurity involves three equally important aspects: (1) safety of the humans and animals in the area, (2) decontamination or disinfection of field equipment, and (3) restriction on transporting amphibians among watersheds.

26.4.1 Human and animal safety

Whenever sampling amphibians for disease, the priority must be personal safety and health. For standard monitoring, biologists should wear gloves and waterproof footwear that can be easily disinfected (e.g. rubber boots). If a die-off is observed, it is important to note whether other vertebrates (e.g. birds, fish) are dead or appear morbid. If so, there is a greater chance the animal deaths are due to toxins, which may present a significant human health risk. In cases with a multiple wildlife taxa die-off, field personnel should leave the site immediately without collecting specimens and notify the nearest public health department and wildlife agency. Persons leaving a multiple-taxa mortality site should wash

and disinfect boots, waders, nets, and field equipment and change clothes before entering a vehicle and leaving the site (discussed in section 26.4.2).

Few infectious diseases of amphibians are contagious to humans. Potential zoonotic diseases that may be carried by amphibians include certain *Salmonella* spp., *Yersinia* spp., *Chlamydothrix* spp. (formerly *Chlamydia*), and some toxin-producing mycobacteria (e.g. *Mycobacterium liflandii*) that can cause skin ulceration. In addition, Gray *et al.* (2007c) demonstrated that *Rana catesbeiana* metamorphs were suitable hosts for the human pathogen *Escherichia coli* O157:H7. We also demonstrated recently that tadpoles could maintain this pathogen in aquatic mesocosms (M. J. Gray and D. L. Miller, unpublished results). Thus, disposable gloves should be worn whenever handling amphibians, and hands washed thoroughly with soap and warm water after removing gloves. In the field, hands can be soaked in a 2% chlorhexidine solution for 1 min or disposable antibacterial wipes used. Avoid exposure of surface water to soaps and disinfectants, as they may negatively affect local flora and fauna. Clothing that becomes stained with feces or skin secretions should be removed as soon as possible and washed in color-safe bleach.

The skin secretions of many amphibians contain potent irritants and toxins. For example, newts (Salamandridae), toads (Bufonidae), and poison-dart frogs (Dendrobatidae) exude toxic skin secretions. Skin secretions of certain newts (e.g. *Taricha*) may cause temporary blindness lasting several hours if the secretions get into the eyes. The parotoid gland secretions of giant toads (*Bufo marinus*), if ingested, can rapidly cause heart malfunction in humans and animals. When handling toads, it is best to avoid touching the parotoid glands. After handling amphibians, avoid touching your eyes or mouth prior to washing hands.

26.4.2 Washing and disinfecting equipment

Cleaning equipment and waders is recommended when leaving any amphibian breeding site, whether it is known that pathogens are present or not (see also www.nwhc.usgs.gov/). Cleaning is a three-step process: (1) washing with a soap or detergent, (2) rinsing thoroughly with clean water, and (3) disinfecting of the objects via a chemical disinfectant. Common soaps or detergents are not disinfectants but are useful in removing sediments and vegetation. Biodegradable soaps should be used in the field and not discarded into surface waters, as many are toxic to amphibians, fish, and invertebrates. Chemical disinfectants need to remain in contact with cleaned and rinsed surfaces for several minutes to kill microorganisms.

Common disinfectants used are chlorhexidine and sodium hypochlorite (bleach). Bleach is often preferred because it is cost effective, easily obtained,

and effective against most bacteria and many viruses. The US Fish and Wildlife Service and American Fisheries Society – Fish Health Section (USFWS and AFS-FHS) (2005) recommend 10 min of exposure of a 0.05% bleach solution (i.e. 28.4 g of 6.15% sodium hypochlorite in 3.8 L of clean water) for disinfection of field equipment and surfaces for *B. dendrobatidis*, and, although not conclusive, a 0.5% solution (i.e. 312 g of 6.15% sodium hypochlorite per 3.8 L of water) is recommended to destroy myxosporeans. However, bleach is not very effective at inactivating *Ranavirus*, and requires at least a 3% concentration (Bryan *et al.* 2009). It should be noted that this concentration can be toxic to amphibians. In contrast, chlorhexidine used at a dosage that is safe for amphibians (0.75% for a 1 min exposure) has been shown to inactivate *Ranavirus* (Bryan *et al.* 2009). Further, it is important to keep in mind that the shelf-life of bleach solutions is influenced by exposure to light, air, and organic material, and solutions should be discarded after 5–7 days. After disinfection, equipment may be allowed to air dry or rinsed with fresh, clean water. Alternatively, if carrying large quantities of water is not possible because multiple field sites are to be visited, surface water from the subsequent site (i.e. where the equipment will be used next) can serve as the rinse water. If mountain systems with stream watersheds are sampled, we recommend that researchers begin sampling at higher elevations and work towards lower sites. If a disease agent is present at higher elevations, it is likely to be at lower elevations due to downstream transmission. Hence, if accidental transmission occurs during travel on fomites, it is less likely to be a novel introduction.

26.4.3 Movement of animals and disease management

Introducing captive-raised or moving wild amphibians into new locations may be necessary because of population declines or extirpations. It is important to understand the initial cause of the die-off to ensure the factor no longer exists. In the case of diseases, environmental testing for the etiologic agent should be done before reintroductions or translocations. For pathogens, existing amphibian species also should be tested to ensure they are not functioning as a reservoir. It hinders conservation efforts to release species with high susceptibility if the pathogen remains at a site. Simultaneously, testing of the source population should be performed prior to reintroduction to avoid introduction of pathogens into the wild. Non-lethal testing as described previously generally can be used. Alternatively, in the case of translocations, lethal testing of common closely related resident species from the donor environment can provide some assurance that the target species is not infected.

Amphibians (dead or alive) from a mortality site should be considered contagious specimens. Morbid animals and carcasses should not be released or

discarded at the same or other sites because this may facilitate the spread or persistence of infectious diseases. Dead amphibians that are not used for testing should be placed in double-layered plastic trash bags and disposed by burial or incineration. Removal of carcasses is a good strategy to help thwart the spread of infectious diseases.

While some serious infectious diseases of amphibians (e.g. *B. dendrobatidis*, nematode lungworms (*Rhabdias* spp.)) are readily treated and eliminated from captive populations, some important infectious diseases have no known treatments (e.g. ranaviruses, alveolates) or no practical treatment in the wild. Treatment of any disease varies by the pathogen involved as well as the host. Some pathogens are resistant to many treatments (e.g. antibiotic-resistant bacteria) and some hosts may be sensitive to a particular treatment (e.g. Methylene Blue may be toxic to tadpoles at concentrations over 2 mg/ml). Generally, it is best to contact a veterinarian with experience in amphibians for proper treatment of disease. However, some treatments (i.e. elevated temperature for *B. dendrobatidis* or dermosporidium, sea salt or Methylene Blue for *Saprolegnia*, chlorhexidine for bacteria and *Ranavirus*) may be attempted by the non-veterinarian and treatment guidelines can be found in Wright and Whitaker (2001) and Poole (2008). As a general rule, treatment for disease is only applicable to captive environments; however, it can be a valuable conservation tool for amphibians slated for release.

In the event that animals destined for release test positive for a treatable disease, the animal and any others that may have been exposed should be treated. Following treatment, a minimum of two negative test results with 1 month between tests should be obtained. If the animal does not test negative, the treatment should be repeated. Only animals that test negative should be released into the wild. In addition, if one animal in a group of 10 housed together tests positive for a pathogen, all of the animals should be treated, regardless of individual test results. Current guidelines for treatment and release have been established by the Association of Zoos and Aquariums (Poole 2008). Testing at the appropriate life stage for the host and disease agent is important.

26.5 Conclusions

Amphibians are declining globally and emerging infectious diseases are one of the causes. Natural resource agencies and conservation organizations should consider establishing amphibian disease surveillance programs that monitor populations for at least the two pathogens linked to catastrophic die-offs: *Ranavirus* and *B. dendrobatidis*. Further, the OIE has listed these pathogens as

notifiable diseases, mandating that *Ranavirus*- and *B. dendrobatidis*-free status be verified prior to movement of amphibians for commerce. Herein, we have provided guidance on collection, storing, and shipping protocol of amphibians to diagnostic laboratories for disease testing. We encourage readers to use the Internet to locate a wildlife diagnostic laboratory in your area.

Given that pathogens can cause significant mortality that have trickle-down effects on ecosystem processes (Whiles *et al.* 2006), biologists must be prudent to decontaminate field equipment and footwear when moving among amphibian breeding sites. We also recommend that natural resource agencies consider implementing wildlife laws that prevent the use of amphibians as fishing bait. Transmission of *Ranavirus* in western North America has been attributed to the movement and sale of *A. tigrinum* larvae (Storfer *et al.* 2007; Picco and Collins 2008). We also encourage natural resource agencies to develop public educational brochures on the threat of amphibian diseases and the benefits of decontaminating recreational gear when leaving watersheds. Finally, prudent land stewardship undoubtedly reduces the likelihood of disease emergence by decreasing the effect of anthropogenic stressors. We encourage support of existing or development of new conservation programs that help landowners establish undisturbed buffers around amphibian breeding sites.

26.6 References

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