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Pathogen Surveillance in Herpetofaunal Populations: Guidance on Study Design, Sample Collection, Biosecurity, and Intervention Strategies

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In the past 20 years, several pathogens have been linked to widespread mortality events of herpetofauna, and in some cases, contributed to population declines and species extinctions (Wake and Vredenburg 2008; Price et al. 2014; Allender et al. 2015a). The reasons for pathogen emergence are diverse, but often are a consequence of human activities. For example, the lack of unregulated trade is resulting in the global translocation of various herpetofaunal pathogens (Auliya et al. 2016). In other cases, anthropogenic factors (e.g., pesticides) may play a role in pathogen emergence by stressing the host and making it more susceptible to pathogen infection and associated diseases (Rollins-Smith 2017). Additionally, pathogen niche modeling with climate change projections show shifts in pathogen and host distributions with possible future conditions (Puschendorf et al. 2009; Murray et al. 2011; Xie et al. 2016). Natural resource and conservation organizations are recognizing the complexity of host-pathogen interactions and the threat that many pathogens pose to herpetofaunal biodiversity (Voyles et al. 2015). As such, the surveillance of pathogens in herpetofaunal populations is becoming more common (e.g., Olson et al. 2013; https://mantle.io/grrs; https://amphibiandisease.org). The goal of this paper is to outline the purposes of pathogen surveillance, possible study designs, approaches to sample collection and biosecurity, and possible intervention strategies that could be implemented to reduce the impacts of herpetofaunal pathogens on host populations.

Surveillance is the process of learning about the occurrence of a pathogen and its effects on host populations in the wild. Often surveillance is performed in the context of determining whether a pathogen is *emerging* (Morner et al. 2002). A pathogen is considered emerging if its geographic distribution is expanding, or if its occurrence at a site or *host range* (i.e., number of species it is known to infect) is increasing (Daszak et al. 2000). Thus, surveillance can involve attempting to detect one or more pathogens in the environment (i.e., environmental DNA [eDNA] sampling), or evaluating host animals for pathogen infection or disease. *Infection* means that the pathogen is alive in the host and often replicating. In contrast, *disease* means the pathogen is impairing the physiology and possibly survival of the host (Morner et al. 2002). Infection and disease should not be used interchangeably. Understanding the relationships between pathogen occurrence at a site and infection or disease in a host is essential to understand possible population and community effects.

All surveillance studies are conducted in some spatial and temporal context, which is often dictated by the project objectives and available resources. Studies that aim to understand the distribution and host range of a pathogen might start with

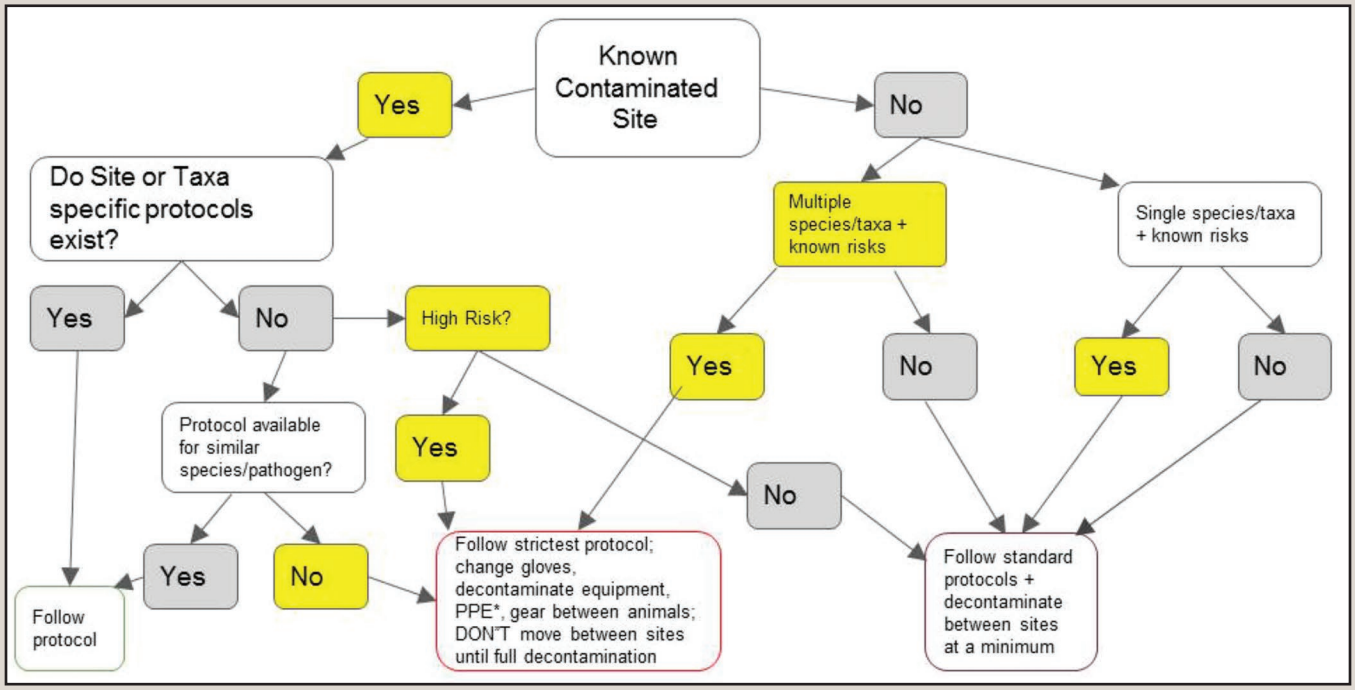

FIG. 1. Simplified decision tree for determining appropriate level of biosecurity warranted when conducting fieldwork. Yellow boxes indicate decision pathways that lead to work within higher risk sites and suggest stricter biosecurity protocols should be followed. Gray boxes indicate either a lower risk (right side of the decision tree), or the existence of an established biosecurity protocol (left side of tree). In general, fieldwork between sites should always start at the lowest risk sites and move to the highest risk sites. If possible, always work at known contaminated sites last, and do not use priority gear (i.e., that which comes in contact with animals) at other sites with lower risk or unknown status. When in doubt, implement the strictest biosecurity protocol possible. *PPE = Personal protective equipment; such as non-powdered vinyl or nitrile gloves, boots or boot covers, coveralls or other clothing that can be sanitized or discarded.

sampling a multitude of sites, but with limited frequency per site (e.g., Hoverman et al. 2012a, b), which is referred to as a cross-sectional design. However, if the objective is to understand disease risk and factors responsible for pathogen outbreaks at specific sites, sampling the same sites frequently (i.e., a longitudinal design) can provide more insight into the epidemiology and impacts of a pathogen. For example, Lips et al. (2006) sampled weekly sites in Panama, and documented the collapse of amphibian communities associated with the emergence of the amphibian chytrid fungus, *Batrachochytrium dendrobaditis* (*Bd*).

Pathogen surveillance usually includes field sampling and subsequent laboratory diagnostic techniques. Pathogens are most commonly identified in samples collected from the field using a laboratory technique called polymerase chain reaction (PCR) that detects the deoxyribonucleic acid (DNA) for a specific pathogen. A quantitative form of PCR (qPCR) estimates the amount of pathogen DNA in a sample, which can be used as a measure of *pathogen load* (i.e., an index of infection intensity). Importantly, PCR and qPCR cannot determine if a pathogen is alive or actively replicating in a host. Thus, if a goal of surveillance is to verify active replication, microbiological techniques (e.g., cell culture) need to be used to show that the pathogen in the host is viable. Serological techniques (e.g., Enzyme Linked Immunosorbent Assay [ELISA]) that detect antigens or antibody responses of the host to the pathogen also can be used to provide evidence of pathogen exposure during a previous infection. If hosts are capable of clearing infections, it is possible to have negative qPCR and cell culture results, but a positive antigen or antibody result in the same host. Hence, serological detection can provide historical context to previous infections. If the goal of surveillance is detection of disease (rather than simply infection), animals typically need to be collected, necropsied, and organ tissues inspected under a microscope to measure indices of organ function (i.e., histopathology). Inspecting animals for gross signs of disease is useful if the signs are *pathognomonic* (i.e., characteristic or diagnostic of a particular disease). For example, ulcerative lesions created by the salamander chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*), and Snake Fungal Disease (SFD), *Ophidiomyces ophiodiicola*, can be diagnostic (Allender et al. 2015b; Van Rooij et al. 2015); however, Miller et al. (2015) warned that many pathogens can produce gross signs that appear to be ranaviral disease.

Infection and disease prevalence are common risk assessment metrics monitored in populations during pathogen surveillance projects. *Prevalence* is an estimate of the percent of individuals detected in a population that are infected or diseased at one point in time. Similarly, *seroprevalence* is the percent of individuals with detectable antigens or antibodies. Estimates of prevalence are useful for addressing basic questions about pathogen distribution and host range; however, a more robust estimate of pathogen risk is incidence. *Incidence* is the number of new cases of infection or disease per unit time, and is calculated by uniquely marking individuals and monitoring their health status in a population over time (Wobeser 2006). Thus, longitudinal data are needed to estimate incidence. Gray et al. (2015a) provided an example of how to calculate and use incidence for inferences on pathogen risk. Pathogen load estimated using qPCR can provide another measure of risk if a large number of pathogen DNA copies in a host is correlated with disease progression, which has

been suggested for *Bd* infections (Vredenberg et al. 2010). Additionally, most pathogens have genetic variation, resulting in different isolates or genetic strains varying with respect to *infectivity* (i.e., ability to infect) or *virulence* (i.e., ability to cause disease). Thus, genetically characterizing pathogens, documenting their occurrence patterns (geographically and in host species), and estimating differences in infectivity and virulence among strains can provide insight into the history and evolution of pathogen emergence (e.g., Goka et al. 2009; Farrer et al. 2011; Schloegel et al. 2012; Rosenblum et al. 2013).

Study Design

The design of surveillance studies will depend on the objectives and implementation constraints. Exploratory studies that aim to understand pathogen prevalence patterns will often start with a cross-sectional design within a geographical area of interest and may focus on one or more host species. Alternatively, surveillance in response to a suspected disease outbreak typically starts with a longitudinal design that involves repeated sampling at target sites, with the goal of identifying factors responsible for the pathogen's emergence.

The first step of a surveillance study is selecting sample sites, which is similar to any species inventory (e.g., Heyer et al. 1994; Fellers 1997). For example, if little is known about the distribution of a pathogen, sample sites can be selected at random using a grid-coordinate system so that inferences can be applied to the broader landscape. If geographical or jurisdictional attributes are of interest, random site selection can be stratified within attributes, such as: 1) governmental jurisdictions (e.g., county, prefecture, state, province); 2) land ownership or land-use allocation (e.g., resource natural area); 3) physiographic or ecological boundaries (e.g., watersheds or ecoregions); 4) categories of habitat quality (e.g., disturbed versus undisturbed sites); or 5) habitat suitability distributions for the pathogen or host species (e.g., Yap et al. 2015; Richgels et al. 2016; Xie et al. 2016). In other cases, it may be more appropriate for sampling to occur at non-random, target locations – for example, when there is a suspected disease outbreak. Non-random sampling can be useful to provide evidence that a particular pathogen is responsible for a mortality event, but inferences on epidemiological parameters, such as infection prevalence, could be biased. Ultimately, the location and extent of sampling depends on the objectives of your surveillance study, knowledge of the host-pathogen system, and resources available.

Table 1. Required sample size to detect a pathogen in a herpetofaunal population with 95% confidence, considering the population size and assumed infection prevalence. Sample sizes were calculated following Ossiander and Wedemeyer (1973), and patterned after Gray et al. (2015a).

	Assumed Infection Prevalence			
Estimated population size	20%	10%	5%	2%
50	5	20	35	50
100	8	23	45	75
250	11	25	50	110
500	13	26	55	130
2000	15	27	60	145
>100,000	15	30	60	150

Required sample size.—After sampling sites are known, the required sample size per site should be estimated. The number of samples that should be collected depends on the objectives of the study and the detectability of the pathogen, which is a function of its prevalence and constraints of the diagnostic methods used. For example, Skerratt et al. (2008) estimated that testing 60 individuals per population was required to achieve 95% certainty of detecting one *Bd*-positive frog using qPCR if infection prevalence in the population was $\geq$5%.

To facilitate sample size calculations, we provide guidance for the minimum required sample size necessary to detect a pathogen based on the host population size and the assumed pathogen prevalence level (Table 1). This is an approach that has been used extensively in fisheries research (Ossiander and Wedemeyer 1973). However, the sample sizes in Table 1 assume perfect detection of the pathogen by laboratory diagnostic techniques. In other words, if qPCR is used to test for occurrence of the pathogen, it is assumed that 100% of the time it will be detected in the sample if the host is infected. This often is not the case because pathogen detection can differ among sample types (e.g., swabs vs. blood) and tissues (e.g., tail clips vs. organs; Gray et al. 2012), and most laboratory diagnostic techniques are imperfect. Thus, it is a good practice to collect more than the minimum sample size in Table 1 to increase the probability of pathogen detection. If the sensitivity and specificity of a diagnostic test are known, prevalence can be adjusted *post-hoc* to reflect a more robust estimate (Rogan and Gladen 1978). *Sensitivity* is the probability of detecting a pathogen when it is present, hence an estimate of avoiding false negatives; whereas, *specificity* is the true negative rate estimated as the proportion of negative test results that are truly negative (Wobeser 2006).

Sample sizes in Table 1 illustrate the effects of population size and pathogen prevalence upon detection, with the latter having the greatest effect. As a pathogen becomes less prevalent in a population, more samples are needed to detect it. Similarly, a smaller sample size is needed to detect pathogens when prevalence is high. The $n = 3/p$ rule is another simple approach to estimating required sample size (n) using the binomial distribution, where p = the lowest prevalence you want to detect (Jovanovic and Levy 1997). Thus, if you want to be 95% confident in detecting a pathogen that is present at a prevalence of 1%, the required sample size is: $n = 3/0.01 = 300$. Similar to the calculations in Table 1, this approach assumes perfect detection by the diagnostic technique used.

The aforementioned approaches for sample size calculations are for detecting a pathogen at a site; however, often the objective of surveillance is to statistically compare two or more estimates of prevalence. Table 2 provides required sample sizes for comparing prevalence estimates, depending on the desired minimum detectable difference between the estimates. For example, suppose that you want to be able to detect a 40% difference in prevalence between two sites, the sample size required to detect this difference is 25–29 per site (i.e., intersection of any two prevalence values in Table 2 with a 40% difference). Program R and several websites (e.g., http://epitools.ausvet.com.au/) have more sophisticated functions for estimating required sample sizes for pathogen detection or comparing multiple prevalence estimates. Program R also can be used to adjust prevalence estimates if sensitivity and specificity of a

TABLE 2. Required sample size per population for detecting differences in infection prevalence between two populations with 95% confidence and 80% statistical power (methods: http://epitools.ausvet.com.au/content.php?page=2Proportions; see Gray et al. 2015a).

Prevalence 1	Prevalence 2										
	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	0.95
0.05	•	474	88	43	27	19	14	11	8	7	6
0.1	•	•	219	72	38	25	17	13	10	8	7
0.2	•		•	313	91	45	28	19	13	10	8
0.3	•			•	376	103	49	29	19	13	11
0.4	•				•	408	107	49	28	17	14
0.5	•					•	408	103	45	25	19
0.6	•				•		•	376	91	38	27
0.7	•			•				•	313	72	43
0.8	•		•						•	219	88
0.9	•	•								•	474
0.95	•	•	•	•	•	•	•	•	•	•	•

diagnostic test are known (e.g., Hyatt et al. 2007; Allender et al. 2013a; 2015c; Blooi et al. 2013).

These approaches to estimating required sample size assume that individuals in a population have an equal probability of capture for sample collection. If infected individuals change their behavior and become more difficult to capture, larger sample sizes will be needed. Similarly, some age classes or species might have different capture probabilities; thus, we recommend that sample size estimates be calculated for each species or attribute of interest (e.g., age, life-history stage, sex). Further, prevalence of many pathogens is known to vary among seasons (e.g., Gray et al. 2007; Kinney et al. 2011), hence sampling >1 season may be necessary to understand pathogen dynamics and host species effects. Gray et al. (2015a) indicated that it might be necessary to sample at least once every two weeks to detect ranavirus outbreaks in wild populations due to rapid progression from infection to clinical disease in some host species. When eDNA is sampled (e.g., via water filtration), both temporal and spatial variation of the pathogen at the aquatic water body warrants consideration during sample collection (e.g., Chestnut et al. 2014; Chestnut 2015).

Interpreting Prevalence Data.—Pathogen prevalence data can be difficult to interpret. Some pathogens exist at high prevalence in populations and infrequently cause disease. For example, high prevalence of *Bd* has been reported in eastern North America with little occurrence of disease (Rothermel et al. 2008). However, the same infectious agent is highly pathogenic to Southern Mountain Yellow-legged Frog (*Rana muscosa*) populations in western North America and many species at tropical latitudes (Lips et al. 2006; Vredenburg et al. 2010). Thus, to appropriately interpret prevalence data, you need an understanding of the disease ecology of each pathogen and host species, the conditions that result in a host species becoming infected and progressing to clinical disease, and what typically constitutes baseline infection prevalence versus prevalence levels during disease outbreaks.

Commonly, controlled laboratory or mesocosm experiments are used to estimate baseline susceptibility of host species to pathogens, and identify the relationship between becoming infected and developing clinical disease (e.g., Carey et al. 2006; Hoverman et al. 2011). A most robust approach to estimate host susceptibility is using a lethal dose (LD)-50 design (Dwyer et al. 1997). An LD-50 study estimates susceptibility by exposing a host species to multiple doses (usually 3 – 5) of the pathogen and estimating the dose where 50% of the individuals die from disease. Similarly, an infection dose (ID)-50 study can provide insight into infection dynamics. LD- and ID-50 estimates can be compared among species or other demographic characteristics (e.g., age class) as a measure of relative susceptibility, and can be used in epidemiological models along with other disease and population parameters to predict the likelihood of an outbreak (Dimmock et al. 2015). Warne et al. (2011) provided a good example of estimating the LD-50 level for Wood Frog (*Lithobates sylvaticus*) larvae exposed to ranavirus. Alternatively, host susceptibility studies can be performed at one dose that has environmental relevance. For example, Hoverman et al. (2011) estimated the susceptibility of 19 amphibian species to ranavirus at one dose (10^3 PFU/mL) that has been reported as an environmentally relevant concentration (Rojas et al. 2005; Hall et al. 2016). Laboratory or mesocosm estimates of the correlation between infection and development of clinical disease can provide insight when interpreting infection prevalence data collected from the field. For example, there is about an 85% correlation between infection prevalence and disease when amphibian hosts (larval stage) are exposed to environmentally relevant concentrations of *Frog virus 3* (FV3)-like ranaviruses (Brunner et al. 2015).

Another way to interpret possible pathogen effects on hosts is to estimate pathogen load in target tissues using qPCR. In general, it is reasonable to assume that as infection load (i.e., pathogen DNA concentration) in a host increases, the likelihood of disease-induced mortality also increases. Several studies have reported very high concentrations of ranavirus and *Bd* in amphibians that succumbed to disease (Kinney et al. 2011; Brand et al. 2016). With some pathogens, there may be an infection intensity threshold that results in a host transitioning from subclinical to clinical disease. Vredenburg et al. (2010) provided evidence that declines in Southern Mountain Yellow-legged Frog populations due to *Bd* generally were not observed unless pathogen load exceeded on average 10,000 zoospore equivalents per swab. Pathogen loads also can be used to make inferences about whether species are resistant, tolerant, or amplification hosts. For example, when a pathogen is never or rarely detected in a species, it might be

resistant, meaning that infection may occur briefly but the host's immune system clears the pathogen quickly. In cases when low-grade, subclinical infections are detected frequently with infrequent occurrence of disease, the host may be tolerant of infection and function as a carrier or reservoir for the pathogen. Alternatively, when high pathogen loads and occurrence of disease are detected, the host may be an amplification species that contributes to rapid pathogen propagation (Paull et al. 2012; Martel et al. 2014). Importantly, no detection of a pathogen or detectable low-grade infection during a sampling event provides little information about the potential epidemiological role of a host, because the pathogen may not be present, conditions may be suboptimal for pathogen emergence, or the sampling event occurred prior to or after the peak of an outbreak. Pathogen load data are most informatively interpreted with host susceptibility results from laboratory or mesocosm experiments.

Understanding baseline prevalence of a pathogen in a population is key to identifying if an outbreak is occurring (i.e., infection levels are above normal). Baseline prevalence estimates can be identified by reviewing the literature (Gray et al. 2015a). If previous studies do not exist, initial surveillance efforts can serve that purpose. Host species often differ in susceptibility to a pathogen; thus, baseline prevalence estimates should be interpreted by species. If community-level prevalence estimates are of interest, they can be averaged among species. For FV3-like ranaviruses, infection prevalence often is very low (<5%) in larval amphibian communities then increases rapidly during an outbreak (Brunner et al. 2015; Hall et al. 2016). This observation and the strong correlation between infection prevalence and disease for FV3-like ranaviruses prompted Gray and Miller (2013) to suggest that infection prevalence exceeding 40% might be an indication that a ranavirus outbreak is occurring. As suggested in Vredenburg et al. (2010), pathogen load also may provide evidence of whether a disease outbreak is imminent. Kinney et al. (2011) provided supportive evidence of the Vredenburg et al. (2010) 10,000-zoospore rule for the average *Bd* infection intensity threshold when chytridiomycosis outbreaks occur. We caution readers about broadly making conclusions about infection thresholds and disease without substantial evidence, because there is tremendous variability in the virulence of pathogens and their interaction with various host species and environmental conditions.

Identifying the Consequences and Factors of Disease Outbreaks.—Pathogens can affect hosts through direct mortality or impairing physiological functions that reduce fitness. The population consequences of disease can range from minimal mortality to gradual or rapid declines. Lips et al. (2006) reported populations of several amphibian species in Panama crashed in <1 year due to the invasion of *Bd*. In northern Spain, it took five years for ranaviruses to cause local extirpation of certain amphibian species (Price et al. 2014). For these studies, the investigators returned to the same sites at least 3x per year to survey amphibians and estimate relative abundance using standard sampling techniques. There are several resources available that discuss effective techniques to capture and monitor herpetofaunal populations (Heyer et al. 1994; Dodd 2010; Graeter et al. 2013; Gray et al. 2013a). These approaches typically differ between aquatic and terrestrial species (Graeter et al. 2013; Gray et al. 2013a), and between common and rare species. For disease monitoring in rare species with low detectability, survey design approaches such as adaptive sampling can improve the probability of host detection (Thompson 2004). If resources exist and sufficient animals can be marked and recaptured, mark-recapture methods can be very effective at detecting and estimating changes in population abundance associated with infection and disease (Williams et al. 2002). Occupancy modeling also has been used to model infection or disease occurrence patterns across the landscape (Adams et al. 2010; Grant et al. 2016a). Importantly, if inferences are going to be made on the occurrence of disease, healthy and diseased animals should be periodically collected and submitted to a veterinary diagnostic laboratory for full health screenings, including histopathology (Whitaker and Wright 2001; Jacobson 2007).

Identifying factors associated with disease outbreaks can be fundamental to conceiving intervention strategies (Johnson et al. 2015). Estimates of population abundance can help determine if density-dependent factors are important. When possible, health parameters that are correlated with fitness or disease should be measured. For example, body size is a common metric measured in amphibian and chelonian disease studies (Allender et al. 2013b; Caseltine et al. 2016). If pathognomonic disease signs are known, they should be recorded. Microhabitat characteristics that are correlated with host survival, or factors that act as stressors, may influence the likelihood of a disease outbreak. For example, water quality can influence the likelihood of pathogen infection for some aquatic species (Gray et al. 2007). Water and air temperature also may be important in creating ideal conditions for pathogens (Berger et al. 2004; Martel et al. 2013; Brand et al. 2016). Prior to beginning a pathogen surveillance study, a literature review should be performed to identify important habitat characteristics for host survival, and what factors are known to facilitate pathogen infection or stress host species.

Cost-Benefit Considerations.—The cost of surveillance is highly dependent on host characteristics (e.g., common vs. rare species), sampling conditions, and geographic location. Costs can include wages for field personnel, sampling equipment and supplies, travel to field sites, and diagnostics. One cost of surveillance that is relatively consistent is pathogen diagnostics. According to the Global Ranavirus Consortium website (http://www.ranavirus.org), the cost for qPCR testing ranges globally from $25–$42 per sample (in 2017 USD). At the University of Tennessee Center for Wildlife Health, for example, $25 per sample (in 2017 USD) is budgeted for qPCR, which covers supply and personnel costs to test for one pathogen with duplicate qPCR runs, creation of a standard curve to estimate pathogen load, and one re-test per sample if needed. Cost for pathogen isolation and histology typically ranges $50 – $100 per sample (in 2017 USD). Budgeting 10% of total qPCR costs is a good strategy to allow for more detailed diagnostics on a subsample of individuals. A common approach is to collect a sufficient sample size to meet surveillance objectives, properly store the samples (discussed later), and if sufficient funds do not exist, perform qPCR on a subset of samples in an attempt to identify trends, which can provide leverage for securing funds to process additional samples.

Biosecurity, Decontamination, and Sample Collection

It is suspected that humans play a major role in the translocation of pathogens among sites (Cunningham et al. 2003), and that sampling populations can lead to outbreaks. Cases exist where researchers are known to be responsible for the introduction of pathogens to populations (Walker et al. 2008). Poor biosecurity practices also can lead to false-positive diagnostic test results (e.g., PCR) if infected samples contaminate uninfected samples. To limit the role in transmitting and moving pathogens within

and among sites and also between samples, biologists and researchers should implement biosecurity protocols as part of surveillance projects. Here, we outline procedures to minimize the risk of cross-contaminating samples and transmitting pathogens between individuals and among sites.

In general, the objectives of a biosecurity protocol are to protect humans, animals, and the environment from pathogens or other harmful biological agents. In a field setting, this includes limiting exposure between personnel and animals as well as among different sampling sites. We suggest that sites are defined by biological population, where the average home range of a host species delineates the population boundary. This recommendation is based on the premise that humans should not translocate pathogens at a greater rate or distance than natural movement of infected animals (Phillott et al. 2010). Home range sizes differ tremendously among herpetofauna worldwide; however, most species have home ranges <10 ha (Wells 2008; Vitt and Caldwell 2014). The center of sampling sites could be used for between-site measurements. Hence, at a minimum, we recommend that between-site decontamination occur if sampling sites are >350 m apart, which is sufficient distance for non-overlapping home ranges between two sites where average home range size is 10 ha.

Another approach to delineating sites could be based on geographic features that are associated with habitat use of a host species, such as wetland depressions, watersheds, or forest and grassland fragments. A general rule of thumb to follow is that biosecurity protocols should be implemented any time personnel enter a vehicle to travel to a different location. Decontamination includes cleaning to remove debris and applying a chemical for a specified duration to inactivate pathogens. Removing debris before chemical application is essential, because soil and organic matter can reduce effectiveness of disinfectants (Hemmingway et al. 2009).

Understanding the potential risk of cross-contamination and translocation of pathogens requires an understanding of transmission pathways and environmental persistence of the pathogen. Given the complexity of transmission potential and out-of-host persistence among herpetofaunal pathogens and variation in the effectiveness of chemical treatments, it is difficult to provide a universal approach to decontamination. Nonetheless, simple procedures can be followed to help reduce the risk of anthropogenic spread of pathogens between animals as well as sampling sites. Below, we provide a review of considerations when implementing a biosecurity protocol as standard procedures of fieldwork. Fig. 1 provides a decision tree as a starting point to help guide planning and establishing decontamination protocols for fieldwork. Additionally, Phillott et al. (2010) provided a good example of several biosecurity considerations for *Bd* given various risk factors.

Within-site considerations.—For most surveillance studies, animals are typically captured and processed for biological information. An exception is pathogen detection using eDNA sampling (Kirshtein et al. 2007; Hall et al. 2016). For eDNA studies, we suggest considering the between-site recommendations in the following section and reviewing Laramie et al. (2015) for suggestions to minimize sample contamination. When biological samples are collected, it is important to minimize stress on captured animals. Many organizations require an animal-handling protocol approved by an Institutional Animal Care and Use Committee (IACUC) to ensure the animal does not experience undue suffering. To minimize suffering, protocols should be put in place to reduce holding and handling time, reduce pain if tissue samples are collected, such as application of a topical analgesic, and estimate the minimum sample size to address the study objectives. In addition to an IACUC protocol, government permits for scientific research may have animal-sampling considerations, and all permits may have requirements for biosecurity and human health hazards. Types of samples collected during surveillance might include: entire animal, tail tips or digits, skin biopsies, skin swabs, shed skin, blood, hemolymph, saliva, venom, gastric contents, and excrement. The choice of sample should align with where the pathogen has the greatest likelihood of infection. eDNA samples might include water, soil, or organic matter (Hall et al. 2016). Procedures for eDNA sampling are reviewed in Pierson and Horner (2016).

Capturing and processing herpetofauna for surveillance studies has the potential to increase the probability of pathogen transmission among infected and uninfected individuals. The goal should be to sample populations without affecting the likelihood of pathogen transmission. Pathogen transmission can increase among individuals by unnaturally increasing contact between individuals (i.e., density-dependent transmission; McCallum et al. 2001), which can occur during capture and processing. Herpetofauna are commonly captured by hand (e.g., area searches, artificial cover objects), in traps (e.g., pitfall, funnel), or using devices (e.g., nets, tongs; Graeter et al. 2013; Gray et al. 2013a). Traps should be checked frequently so capture densities do not exceed those in the wild and thus artificially increase the risk of exposure to pathogens between individuals. Similarly, capturing individuals by hand or using devices can change transmission probabilities if individuals are housed together in containers (e.g., bag, bucket; Fig. 2). Individuals should be placed into different holding containers upon capture, or when immediate isolation is not possible, such as with many traps or nets, individuals should be placed into different holding containers as soon as possible after capture (Fig. 2).

Standardized protocols for handling animals during processing and sample collection should be established to minimize biosafety risks to animals and personnel. Ideally, researchers should wear non-powdered vinyl or nitrile exam gloves and change them between each animal that is handled (Cashins et al. 2008; Mendez et al. 2008; Greer et al. 2009). Discarding gloves after handling each animal produces a lot of waste and is expensive; thus, dipping gloved hands into disinfectant between animals, allowing sufficient contact time of the disinfectant, and rinsing with clean water is another option (Fig. 2). There is some evidence that processing animals with bare hands and washing between them can reduce transmission of *Bd* (Mendez et al. 2008); however, the effectiveness of this practice for other herpetofaunal pathogens is unknown. Also, although pathogens might become inactivated by disinfecting gloves or washing hands between animals, pathogen DNA can remain which can increase the likelihood of cross-contaminating samples if PCR is used as a diagnostic technique. Another option is to use a secondary container or device to handle the animal. For example, a plastic bag can be wrapped around a gloved hand to pick up an individual, or small aquarium nets that can quickly be disinfected and rinsed can be used to move aquatic animals captured in nets or traps to individual containers. The prudent strategy is to assume that each individual that is handled is infected and will result in 100% transmission if an uninfected individual is handled subsequently.

Once captured animals are in individual containers, they should be taken to a processing station where biological data of

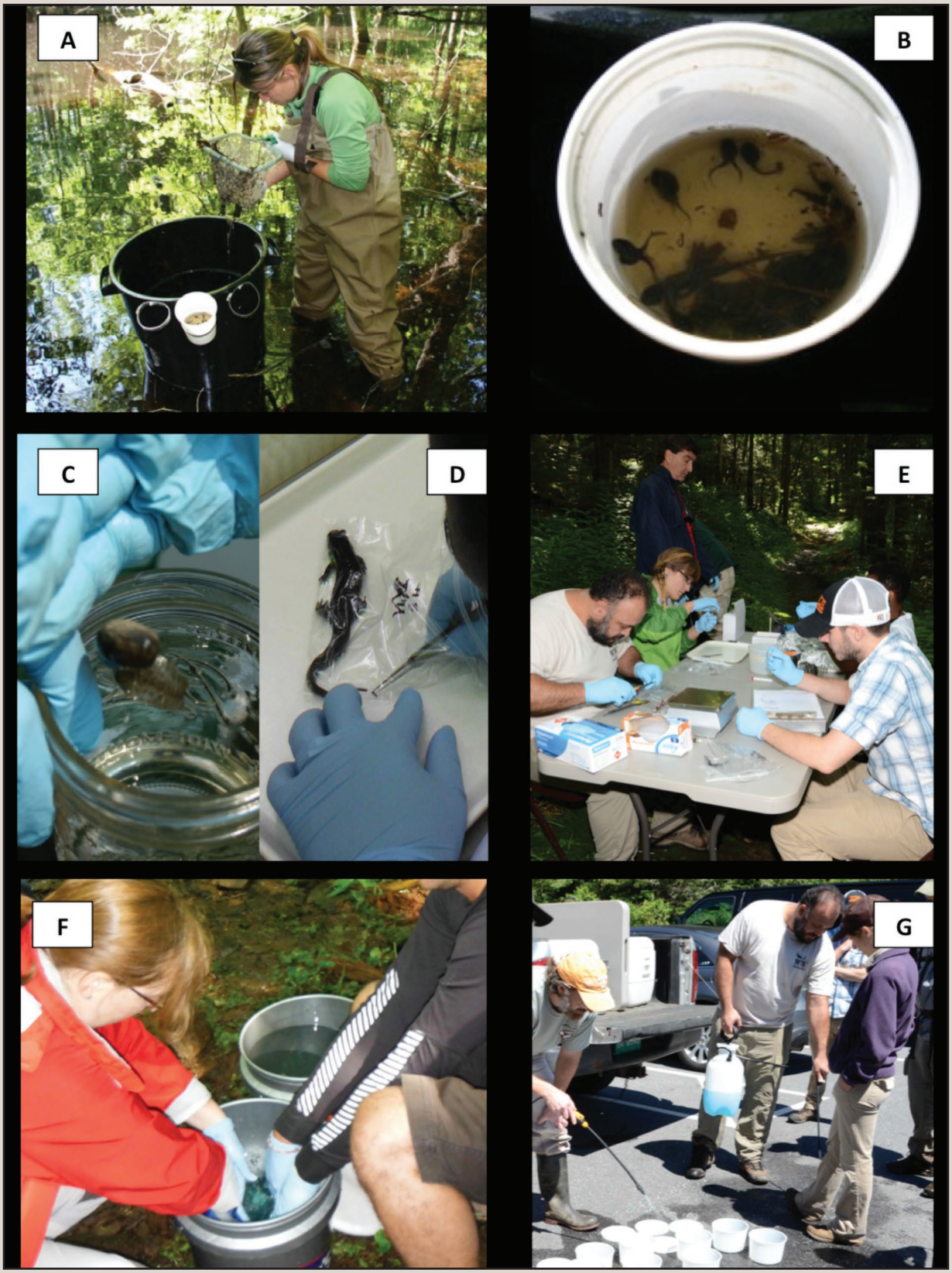

FIG. 2. Pathogen surveillance typically includes capturing animals, processing them individually on an aseptic station, and decontamination. A) Enclosure sampling for aquatic amphibians. B) Captured animals should not be housed together during processing as seen here. C) Each animal should be placed in individual containers. D) Non-lethal samples (e.g., tail clips) often can be collected without touching the animal. E) Station should remain aseptic, with everyone changing gloves between animals; individuals that process should not collect animals. F) Individuals that capture animals should disinfect or change gloves between handling animals. G) All equipment, footwear, and containers must be disinfected before leaving the sampling site.

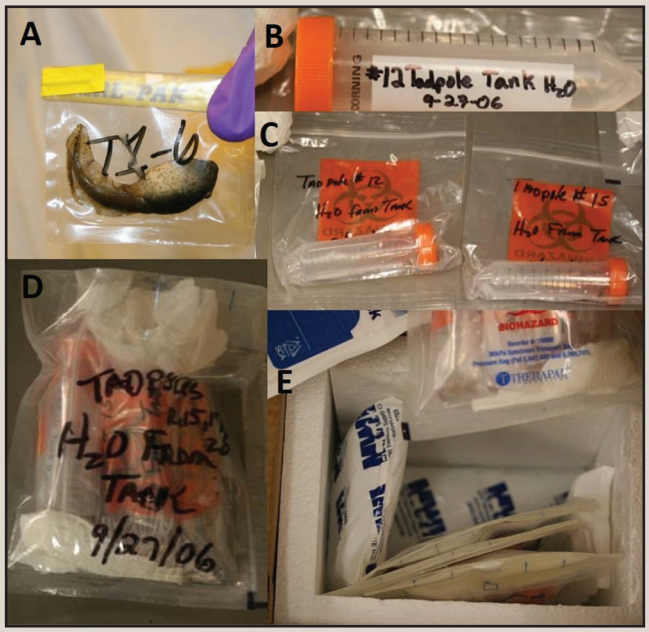

FIG. 3. Example of triple package for shipping samples to a diagnostic laboratory. A) and B) Samples are placed in appropriate leak-proof containers (layer 1). C) Primary containers containing the sample are placed in a secondary leak-proof container (layer 2). D) Absorbent paper is placed within all but the primary container. E) A Styrofoam container (layer 3) serves as an appropriate cooler for shipping with appropriate numbers of ice packs and absorbent paper, but must be placed within a cardboard shipping box.

interest (e.g., weight, length) are collected and samples taken for pathogen testing. It is best to use a processing station that is a flat, ergonomic surface (e.g., portable table) that can be easily disinfected (Fig. 2). For pathogen testing, all captured individuals should be processed to ensure that a representative sample of the population is collected. If this is not feasible, individuals can be randomly selected for processing. We do not recommend processing animals systematically (e.g., every third one) or haphazardly (i.e., researchers selecting individuals nonchalantly), because these approaches can lead to biased prevalence estimates. When possible, researchers that process animals should not participate in capture efforts, as this may increase the risk of contaminating samples. Ideally, we recommend that one individual be assigned to each processing task. For example, one individual could collect morphometric data from the host (e.g., body size), and a different individual collect samples for pathogen testing. If multiple samples are collected (e.g., swab, biopsy, blood), a different individual could be assigned to each task if sufficient personnel exist. After processing, animals should be returned to their capture location.

Between-site considerations.—Environmental conditions and the presence of suitable hosts will impact whether pathogens are present at a site. Historically, researchers focused on one pathogen for surveillance (e.g., Lips et al. 2006), which commonly led to consideration of only the target pathogen and hosts when sampling. More recently, surveillance efforts have expanded to including multiple pathogens (e.g., Hoverman et al. 2012b; Souza et al. 2012), because co-infections can occur and possibly cause different disease outcomes compared to if only one pathogen is present (Miller et al. 2008; Rynkiewicz et al. 2015; Sim et al. 2016). Even if the scope of the study is limited to a single pathogen and host species, investigators should consider the environments that they will be traversing, and pathogens that could be translocated to other sites. For example, if working with reptiles primarily in xeric environments, researchers may not typically think of pathogens in aquatic systems; however, care should be exercised to prevent spread of non-target pathogens if water bodies are traversed during sampling. Similarly, amphibian researchers may be focused on chytrid fungi and ranaviruses, but may visit sites with SFD without considering disinfectants that can inactivate *Ophidiomyces*.

If disease risk is known at multiple sites, researchers should sample in the order of low to high risk sites. We define high-risk sites as those where a novel pathogen is present, where endemic pathogens are at high prevalence, or where there are known disease outbreaks. For example, Sutton et al. (2015) reported that ranavirus prevalence in plethodontid communities was greater at low compared to high elevation sites, hence future sampling order of those sites should occur from high to low elevation.

All sampling gear and footwear should be cleaned and disinfected between sites. If only a few sites are sampled and resources exist, researchers could consider having different sets of gear for each site, while still following a disinfection protocol after sampling at each site. Equipment used for sampling herpetofauna might include measuring and weighing devices, holding containers or tubes, tongs, traps, nets, waders, marking devices (e.g., PIT tag injector, shell-notching files), scissors, scalpels, etc. (Gray et al. 2013a). Equipment also might include devices for measuring habitat quality (e.g., water quality meters, temperature loggers). Gear that is nonporous, non-corrosive, easy to clean and disinfect, or is single use (e.g., plastic bags) is ideal. It is especially important to clean and disinfect any gear that comes into contact with an animal before use with another individual.

A scrub brush and water can be used for initial cleaning to remove all soil and organic matter that is attached to equipment or footwear, which should be done until they appear to be visually clean. Thereafter, an appropriate disinfectant should be applied to all surfaces for the required minimum contact duration (Appendix I). Hand-pump sprayers, such as those used for herbicide application, can facilitate application (Fig. 2). For some disinfectants (e.g., bleach), clean water can be applied after the appropriate contact time to reduce negative effects on equipment. When sampling at very high-risk sites, disposable gowns (e.g,. Tyvek®) may be appropriate. It is possible that some pathogens could be transported from site to site on vehicle tires or boats. If that possibility is suspected, especially in high-risk scenarios, spraying disinfectant on vehicle tires, boats and trailers between sites is a good practice. Taking vehicles to car washes where wastewater enters a sewage treatment facility has been used as a biosecurity measure for invasive plant and animal species to forestall inadvertent translocation, and may have similar effects on pathogens.

Chemicals for disinfecting.—Appendix I lists chemicals commonly used for disinfecting equipment, and provides information on efficacy for major pathogens as well as potential human health and environmental risks, and options for mitigating these effects. Concentrations of 3% bleach (active ingredient [AI] = 6% sodium hypochlorite), 1% Virkon® Aquatic (AI = 21.4 % potassium peroxymonosulfate), and 1% Nolvasan® (AI = 2% chlorhexidine diacetate) will inactivate most pathogens with 1-minute contact time (Johnson et al. 2003; Bryan et al. 2009; Gold et al. 2013). Some pathogens require greater concentrations or contact time with disinfectants. For example, 10% bleach with 2-minute

Table 3. Examples of appropriate samples to take for some common pathogens of concern in various herpetofaunal hosts.

Host	Pathogen	Sample
Amphibians	Ranavirus	Liver, kidney, lesions
	Batrachochytrium dendrobatidis *B. salamandrivorans*	Keratinized skin (especially lesions, toes, tooth rows of tadpoles)
Chelonians	Ranavirus	Respiratory mucosa, oral mucosa, lung, blood, lesions
	Mycoplasma	Lung, respiratory mucosa
	Herpesvirus	Lung, respiratory mucosa
Squamates	Snake Fungal Disease	Skin lesions

contact time is recommended to inactivate *Ophidiomyces ophiodiicola* (Rzadkowska et al. 2016). In cases where sampling locations are remote, granulated bleach or Virkon® can be mixed with surface water and used prior to leaving a site.

When applying chemical disinfectants, users need to take care of human risks and follow any regulations. For most organizations, users should be trained in applying and be briefed about the chemicals in disinfectants and potential human and animal health hazards. At a minimum, it is important that all personnel: 1) read the Safety Data Sheet for the disinfectant and active ingredients; 2) use appropriate personal protection; 3) know how to properly apply the disinfectant, especially required concentrations and contact times; 4) know how to collect and dispose of the chemical; and 5) know appropriate emergency response and contact information in case of harmful personal exposure.

Sample Storage and Uses

Pathogen surveillance samples in herpetofauna can be collected non-lethally or lethally. To make a decision on an appropriate sample type to collect, knowledge of tissues that pathogens target is needed. Unfortunately, there is no universal sample type that detects infection or appropriately diagnoses every disease in herpetofauna. *Batrachochytrium dendrobatidis* infects keratinized tissue; thus, a sample of an internal organ is not useful. In contrast, ranavirus is commonly found in the blood and in liver, spleen, and kidney tissue of hosts. To test for *Bd* and ranavirus simultaneously in post-metamorphic amphibians, a toe-clip sample can be used. In chelonians, ranavirus is a systemic disease, whereas herpesvirus and *Mycoplasma* (pathogens with similar disease signs) are restricted to the respiratory epithelium. A sample of respiratory epithelium would be needed to diagnose all three pathogens in a single sample. *Ophidiomyces* is restricted to the skin in vipers, but may become systemic in non-venomous species (Allender et al. 2015a). Sampling the skin of vipers has higher detection probability at the lesion, thus in SFD suspect cases, a thorough physical examination of the animal is needed, because sampling distant from the lesion increases the false negative test rate (Allender et al. 2016).

In general, we recommend non-lethal samples be collected unless diseased animals are observed, with the caveat that, depending on the pathogen, most non-lethal sampling techniques may result in a moderate number of false-negative test results. Gray et al. (2012) found that swabs and tail clips from anuran larvae resulted in 20–22% fewer positive cases of ranavirus infection when compared to liver samples. Non-lethal samples for pathogen testing generally include swabs, tissue, and blood. We recommend that researchers consult the current literature or a veterinary diagnostic laboratory to inquire about the best non-lethal sampling technique for a target pathogen and host, but some examples also are provided in Table 3.

Contacting the laboratory that will be testing surveillance samples before sampling is important to understand what to collect and how to store and transport samples; however, some general guidelines follow. For individuals that are suspected to be diseased, "fresh is best." Although transport or shipping of live animals may be possible, it is best to confirm with the diagnostic laboratory that live animals can be received. Generally, most laboratories prefer that live animals are euthanized and shipped overnight with ice packs. Techniques for humanely euthanizing herpetofauna are provided by the American Veterinary Medical Association (AVMA) and can be accessed on the AVMA website: https://www.avma.org/KB/Policies/Pages/Euthanasia-Guidelines.aspx. When fresh specimens or tissues cannot be tested immediately or sent overnight to a laboratory, they may be frozen at -20 or -80°C, or preserved in ≥ 70% ethanol (EtOH) or 10% neutral buffered formalin. If whole animals or organ tissues are collected and preserved, we recommend collecting duplicate samples from each individual, freezing one and preserving the other in formalin. Formalin is generally better for evaluation by histopathology and freezing is better for pathogen culture, whereas freezing or ethanol is ideal for PCR. Table 4 provides guidance on the uses of sample types given the storage method. For most surveillance studies, preservation in ethanol may be ideal because samples do not need to remain cool in the field.

Shipping Samples to a Diagnostic Laboratory

Shipment of fresh or frozen samples should occur overnight and in accordance with courier-specific guidelines, and national and international regulations. In some countries, shipment of possible host species of a pathogen or their tissues is not allowed without a permit. For example, Switzerland placed a ban on the import of salamanders due to the threat of *Bsal* (Gray et al. 2015b). For preserved specimens, overnight shipment is unnecessary. General guidelines for shipment include triple packaging and labeling each layer of packaging with a waterproof writing utensil (Fig. 3). Commonly, the first package layer is the specimen or sample in a sealable plastic bag, tube or container. The second layer is a larger sealable plastic bag in which multiple

specimens or samples can be placed. If the first package layer contains liquid (e.g., ethanol), animals or tissues, absorbent material (e.g., paper towel) should be added to the second package to absorb contents if a leak or spill occurs in the first package. The third packaging layer typically is a padded box or shipping cooler, which is generally placed within a cardboard box. Soft-sided coolers are not appropriate for shipping. For fresh specimens, adequate ice packs should be added around the secondary package. Dry ice should be used with caution. It is best to avoid using dry ice with fresh or fixed specimens because tissues may freeze and prevent histological analysis. For frozen specimens, ice packs or dry ice can be used. The package should contain a detailed list of all contents, a description of requested services, and the contact information of the shipper. This should be placed in a waterproof sleeve (e.g., sealable plastic bag) and placed on top of the tertiary container (i.e., cooler), yet within the outer cardboard box. We recommend calling the diagnostic laboratory prior to shipping for specific instructions. It also is recommended to request a specific contact person (e.g., the pathologist or other person who will be reporting your results) so that you can email them the tracking information directly. In some cases, you may want to email a data file for entering diagnostic results and for cross-checking the sample identification codes.

Shipping specimens with ethanol, formalin or dry ice is regulated in most countries. In the USA, if < 500 mL (< 30 mL per container) of ethanol or formalin are shipped, a "Dangerous Goods in Excepted Quantity" label must be on the package. If > 500 mL of these liquids is shipped, a Class III "Dangerous Goods Declaration" is required. Also, no more than 2.26 kg of dry ice can be used in the USA. Countries and couriers differ in their procedures for labeling and packaging these substances, so check their guidelines prior to shipping. In addition, packages should be labeled, "Exempt Animal Specimen." If specimens are fresh or frozen, "Refrigerate Upon Arrival," should be indicated on the outside of the package. Shipping restrictions may change over time; thus, it is important to check with the shipper for the most current shipping guidelines. Additional information regarding packaging and shipping of diagnostic specimens can be found on the U.S. Centers for Disease Control (http://www.cdc.gov/), U.S. Geological Survey (http://www.nwhc.usgs.gov/), and the Partners in Amphibian and Reptile Conservation (PARC; http://www.parcplace.org) websites, as well as others.

Intervention Strategies

Often a goal of surveillance studies is to identify factors responsible for a disease outbreak and to be in a position to implement intervention strategies that reduce population impacts and thwart future pathogen emergence. Intervention strategies generally fall into three categories: 1) prevent entry; 2) stop or reduce host-pathogen transmission; and 3) remove or reduce a stressor (Woodhams et al. 2011, 2016; Grant et al. 2016b). In cases where a pathogen is not present and introduction is deemed a serious risk, all routes of possible entry into the population should be identified, and sufficient hosts tested at a routine frequency to conclude that the site has a high probability of being pathogen free. Gray et al. (2015a) discussed an example of designing a risk analysis for entry of ranavirus into a site following a protocol established by the World Organization for Animal Health. As presented in the preceding section, disinfection is a biosecurity measure to forestall inadvertent human-mediated pathogen transmission (Fig. 2).

If a pathogen gains entry to a new geographic area or is already present in the environment and is emerging in response to natural or anthropogenic factors, it may be desirable to implement intervention strategies (Woodhams et al. 2011; Scheele et al. 2014). Some natural factors can cause stress and can facilitate transmission, such as when animals tend to cluster (e.g., breeding, estivation, hibernation). In general, these are natural processes that should not be interrupted unless a novel pathogen is introduced that is deemed very high risk. Commonly, herpetofaunal habitats surrounded by anthropogenic land uses are locations of pathogen emergence. For example, elevated prevalence of some amphibian pathogens has been reported in wetlands used by cattle (Johnson and Lunde 2005; Gray et al. 2007). Livestock can affect sites by decreasing water quality and reducing vegetation (Schmutzer et al. 2008; Burton et al. 2009). Poor water quality can stress amphibians, which could increase the likelihood of pathogen infection and disease. Reductions in natural vegetation could affect pathogen dynamics two-fold, by: 1) increasing contact rates (hence pathogen transmission) among individuals, because habitat use of many herpetofauna is concentrated to areas with vegetative cover (Greer and Collins 2008); and 2) changing ambient or water temperatures. Thus, strategies that reduce livestock access to aquatic habitats, such as rotational grazing, exclusion fences, or providing water troughs might reduce pathogen transmission. Vegetation buffers around herpetofaunal habitats are another disease mitigation strategy, which can filter contaminants in run-off prior to them entering aquatic sites. Vegetation also helps maintain more natural diurnal fluctuations of temperature in herpetofaunal habitats. The pathogenicity of many disease agents and immune responses of ectothermic vertebrate hosts is related to temperature. Typically, immune function of ectothermic vertebrates increases with temperature, whereas the growth rate of pathogens often has a temperature optimum. Removal of vegetation usually results in increasing microhabitat temperatures, which can reduce the pathogenicity if the host has a higher thermal tolerance than the pathogen (Nowakowski et al. 2016). Conversely, higher temperatures may benefit pathogens. For example, some ranaviruses replicate faster as temperature increases (Ariel et al. 2009). Excessive water or ambient temperatures also can stress animals and potentially lead to pathogen outbreaks (Brand et al. 2016). The key to devising an intervention strategy associated with a stressor is first identifying if a stressor is responsible for or a contributing factor to an outbreak, and then devising ways to lessen its effects.

To reduce transmission between hosts and pathogens requires knowledge of the pathways of infection. Transmission of many pathogens is density dependent (McCallum et al. 2001); thus, reducing animal density can decrease the likelihood of an outbreak. Strategies such as increasing habitat quantity or quality can help disperse individuals. For example, if snake hibernacula are a limiting factor and a primary location for SFD transmission, constructing additional hibernacula might be a reasonable intervention strategy. Robust stands of emergent vegetation in wetlands are also believed to reduce the transmission of ranavirus (Greer and Collins 2008) and likely other pathogens associated with aquatic herpetofauna. In cases where the possibility of pathogen spillover to adjacent populations is considered unacceptable, euthanizing individuals may be a reasonable strategy for common species. For repatriation projects, Pessier and Mendelson (2010) discussed approaches to ensure that infected captive animals are not released into the wild, which is

TABLE 4. Uses and limitations of various sample types that can be collected during surveillance sampling for herpetofaunal pathogens. Preserved swab and tissue samples are ideally stored dry or in >70% EtOH, while 10% buffered formalin is ideal for preserving whole animals. PCR = polymerase chain reaction; ELISA = Enzyme Linked Immunosorbent Assay.

Sample	Test	Information	Limitation
Frozen swab or tissue (e.g., blood, tail or toe clip, scute, scale)	PCR, pathogen culture, sequencing of cultured pathogen or PCR product	Detect pathogen DNA or RNA and phylogenetics	Low DNA or RNA (often an issue with swabs) may cause false negatives; disease not diagnosed
Preserved swab or tissue (e.g., blood, tail or toe clip, scute, scale)	PCR, sequencing of PCR product	Detect pathogen DNA or RNA and phylogenetics	Low DNA or RNA (often an issue with swabs) may cause false negatives; disease not diagnosed
Live or freshly dead animal (fresh/not frozen)	PCR, pathogen culture, sequencing of cultured pathogen or PCR product, histology, cytology, hematology, toxicology, parasitology	Infection and disease can be diagnosed; full list of health differentials	Euthanasia may be required, rapid shipment with ice
Live or freshly dead animal (frozen)	PCR, pathogen culture, sequencing of cultured pathogen or PCR product; limited histopath for reptiles (generally not possible for amphibians)	Detect pathogen DNA or RNA and phylogenetics	More pathogen DNA or RNA than swabs or tissue, which aids in culturing and sequencing. Freezing ruptures cells so the presence of disease often cannot be diagnosed.
Live or freshly dead animal (preserved)	PCR, sequencing of PCR product, histology, parasitology	Detect pathogen DNA or RNA, phylogenetics, and disease can be diagnosed	Euthanasia may be required, cannot culture pathogen
Blood from live animal	PCR, pathogen culture, sequencing of cultured pathogen or PCR product, hematology, toxicology, parasitology, ELISA	Detect pathogen DNA or RNA and partial list of health differentials, detect past exposure (antibodies) to some known pathogens	Disease generally cannot be diagnosed, drawing sufficient quantities of blood from herpetofauna can be difficult

known to have occurred previously (Walker et al. 2008). Sim et al. (2016) provided a good example of isolation and treatment strategies for a ranavirus epizootic in captivity.

Case studies in the field.—Monitoring herpetofaunal populations and estimating infection or disease prevalence are important to understand host-pathogen epidemiology. However, research into intervention strategies is a necessary complement if conservation is a goal. Below, we present some intervention strategies for three different pathogens that have worked or might work in field situations.

Batrachochytrium dendrobatidis: Several intervention strategies against *Bd* have shown promise in the field. An antimicrobial chemical was used to clear *Bd* from Mallorcan Midwife Toads (*Alytes muletensis*) and from their aquatic habitat in one study area on the island of Mallorca (Bosch et al. 2015). The methods involved applying Virkon® S (DuPont) to the aquatic environment and surrounding terrestrial habitat. This approach might be useful in eradicating an emerging pathogen that is found in an isolated location and considered a novel introduction and high risk (e.g., *Bsal*). However, widespread use of Virkon®, which also kills beneficial bacteria and fungi in the ecosystem, generally is not practical or desirable over a wide geographical area. In another study, Cascades Frogs (*Rana cascadae*) were treated with the anti-*Bd* fungicide itraconazole, which reduced *Bd* on the frogs for at least five weeks and was associated with improved over-winter survival (Hardy et al. 2015). Chestnut (2015) compared several fungicides, which varied in their efficacy of *Bd* treatment of Pacific Treefrogs (*Pseudacris regilla*).

Probiotics have been used successfully in the field to protect a population of Southern Mountain Yellow-legged Frogs from *Bd* in the Sierra Nevada Mountains. Based on successful laboratory trials (Harris et al. 2009), a field experiment was conducted using bioaugmentation of an anti-*Bd* probiotic *Janthinobacterium lividum*, which was found naturally on some of the frogs. Frogs treated with a probiotic bath had lower peak infection loads than untreated controls during the first year of the experiment. In year two, untreated controls were not recovered, whereas 39% of probiotic-treated individuals were recovered (Vredenburg et al. 2011; Bletz et al. 2013). These results suggest that the probiotic treatment allowed individuals to survive by preventing *Bd* from reaching a lethal threshold. The probiotic *J. lividum* has not worked in all cases (Becker et al. 2011) so efforts that focus on optimizing probiotic selection are important (Bletz et al. 2013).

There is some evidence of an adaptive immune response to repeated *Bd* exposures. McMahon et al. (2014) showed that Cuban Tree Frogs (*Osteopilus septentrionalis*) that were exposed to live or dead *Bd* had lower *Bd* loads on the skin during subsequent exposures. In addition, the number of previous exposures to *Bd* was a positive predictor of lymphocyte abundance, suggesting a role of the adaptive immune system in protecting against *Bd*. This strategy could be important in repatriating individuals from survival assurance colonies to the wild. In a recent study, Sierra Nevada Yellow-legged Frogs (*Rana sierrae*) in populations persisting with *Bd* were less susceptible to infection in laboratory trials than individuals from populations without prior *Bd* exposure (Knapp et al. 2016). This result was explained by a number of non-mutually exclusive factors, such as selection for more effective antimicrobial peptides or bacterial defenses, but could also be explained by acquired immunity. These studies suggest that further research into acquired immunity and vaccine development may be fruitful.

Snake Fungal Disease: To date, reported treatment of SFD with antifungal compounds has been unsuccessful in the Eastern Massasauga (*Sistrurus catenatus catenatus*; Allender et al. 2011; Tetzlaff et al. 2015). Newer treatments are being developed and show promise. For example, terbinafine administered through nebulization or subcutaneous implant was shown to maintain proposed therapeutic levels in healthy Cottonmouths (*Agkistrodon piscivorus*), but needs investigation in infected snakes (Kane et al. 2017).

Environmental manipulation may be possible as well. *In vitro* studies of *Ophidiomyces* demonstrated a thermal gradient of growth, with 25°C leading to optimal growth. Growth is inhibited at 7°C and minimized at 35°C (Allender et al. 2015a). Many host species of *Ophidiomyces* cannot tolerate these thermal extremes, but temperature therapies might be possible in certain species. As one possibility, making clearings in forests could create high temperature refugia from diseases (Puschendorf et al. 2009; 2011). However, the ability of *Ophidiomyces* to remain alive, but dormant, at extreme temperatures remains to be evaluated. More studies are needed on intervention strategies for SFD, including the role of the microbiome, volatile organic compounds (Cornelison et al. 2014), and pharmacodynamic studies using anti-fungal compounds.

Ranavirus: There are currently no consistently effective treatments for ranavirus infections, although several of the therapeutic recommendations provided for *Bd* and *Ophidiomyces* could be beneficial. Ranaviruses are known to replicate between 12°C and 32°C (Chinchar 2002; Ariel et al. 2009), hence treatments or intervention strategies that modify the body temperature of hosts beyond these temperature extremes might inactivate the pathogen (Sim et al. 2016). The efficacy of temperature manipulations is likely dependent on host and *Ranavirus* species, and possibly age class. Brand et al. (2016) reported greater pathogenicity of an FV3-like ranavirus at 25°C compared to 10°C, 12°C and 15°C in Wood Frog (*Lithobates sylvaticus*) larvae, while Allender et al. (2013b) reported lower pathogenicity of an FV3-like ranavirus at 28°C compared to 22°C in Red-eared Sliders (*Trachemys scripta elegans*).

Vaccines for ranaviruses are in the early stages of development. A yeast-derived vaccine was developed for Chinese Giant Salamander (*Andrias davidianus*) iridovirus (Zhou et al. 2015). DNA vaccines have been used to treat other iridoviruses (Caipang et al. 2006; Zhang et al. 2012). Antiviral medicines, such as acyclovir, valacyclovir or famciclovir, also may be useful (Johnson 2006; Allender et al. 2012; Sim et al. 2016). Exposing Chinese Giant Salamanders to inactivated ranavirus reduced mortality when exposed later to viable virus (Liu et al. 2014). Juvenile and adult amphibians generally have robust immune responses to ranavirus infections (Grayfer et al. 2015), hence exposure to inactivated virus may help mount a secondary adaptive immune response. The adaptive immune response of larval amphibians, however, is weak (Grayfer et al. 2015); therefore, this therapy is likely age-class dependent. Also, adults of some host species seem to be unable to fight ranavirus infections (Cunningham et al. 2007; Balseiro et al. 2009; Sutton et al. 2014). Other treatments for ranavirus infections might include use of molecular tools that can permanently change the pathogen's DNA and thus impact downstream physiological functions. Such technology includes DNA aptamers or clustered regularly interspaced short palindromic repeats (CRISPR; Li et al. 2014; Dong et al. 2016); however, further investigations are warranted before field use of these novel tools.

It is unknown if probiotics would be a useful treatment for ranavirus-infected animals. Chinchar et al. (2004) noted that the antimicrobial properties of amphibian skin reduced ranavirus titers; however, it is unknown if these properties would translate to increased host survival. Unlike *Bd*, ranaviruses infect hosts through multiple routes, including the epithelial cells of the skin, gills and intestines (Miller et al. 2015). Hence, although antimicrobial properties of the skin may afford some protection to ranavirus, infection can still occur if a host is exposed through other pathways, which is likely in aquatic environments or if infected hosts are ingested via predation or necrophagy.

The application of disinfectants in isolated aquatic systems may be effective at inactivating ranavirus. Virkon®, Nolvasan®, bleach, and ethanol are known to inactivate ranavirus (Bryan et al. 2009; Gold et al. 2013). However, as warned, widespread application of disinfectants in the wild is not encouraged due to the negative impacts on native biota. The environmental persistence of ranavirus in aquatic systems is likely between 1–3 weeks, depending on temperature and the presence of microbiota, as microbiota appear to reduce ranavirus persistence (Johnson and Brunner 2014; Munro et al. 2016). Therefore, strategies that facilitate occurrence of natural microbiota in aquatic ecosystems likely will help reduce the persistence of ranavirus. Desiccation also is known to inactivate ranavirus (Brunner et al. 2007); thus, in managed wetland or lake systems with water control structures (Gray et al. 2013b), performing a complete drawdown could reduce ranavirus persistence if the soil dries completely.

Ranaviruses, and many other pathogens, are known to coevolve with their host (Brunner et al. 2015). Teacher et al. (2009) demonstrated that the survivors of a population of Common Frogs (*Rana temporaria*) that experienced a ranavirus die-off had greater frequencies of Major Histocompatibility Complex (MHC) Class I alleles, which probably occurred through positive selection. Expression of MHC Class I genes is known to contribute to ranavirus immunity (Grayfer et al. 2015). Thus, it may be possible to produce immunologically resistant individuals in captive populations (Woodhams et al. 2011) by exposing them to ranavirus isolates. Selection for MHC genotypes has been shown for other amphibian pathogens (e.g., Savage and Zamudio 2011, 2016). This strategy might be used to increase the success of repatriation following loss of a species from a site when the pathogen remains present. Creating populations of immunologically superior individuals in livestock breeding is not novel (Mallard et al. 2015; Sivaraman and Kumar 2015); however, its success in wildlife populations is less understood.

Conclusions

Performing a pathogen surveillance study requires significant planning and effort to yield useful results, while minimizing the risk of pathogen transmission between infected and uninfected animals, cross-contamination among samples during processing, and translocation of pathogens among sites. In addition to developing a strong experimental design and acquiring sufficient sample sizes, strict biosecurity practices should be followed. For individuals that have not previously performed surveillance studies, we recommend that disease experts be consulted. Many organizations (e.g., PARC, Association of Reptilian and Amphibian Veterinarians, Global Ranavirus Consortium) can provide guidance or connect you with professionals that have substantial experience in pathogen surveillance. Recently, PARC formed a National Disease Task Team (http://parcplace.org/parcplace/

resources/disease-task-team.html) to facilitate communication among professionals and organizations tasked with monitoring and responding to the emergence of infectious diseases in herpetofaunal communities. Establishing collaborations and partnerships is key to documenting the distribution of emerging pathogens, identifying routes of entry or sites of concern, and implementing strategies that reduce the likelihood of pathogen spread and disease outbreaks.

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Appendix I

Recommended disinfectants for use in decontamination protocols for reptile and amphibian field studies. [1]Bryan et al. (2009); [2]Horner et al. (2016); [3]Pessier and Mendelson (2009); [4]Phillott et al. (2010); [5]Rocky Mountain National Park (2009); [6]Rzadkowska et al. (2016); [7]Samora et al. (2012); [8]USFS (2017); [9]USFS (2005); [10]WDFA (2012); [11]Rzadkowska, M. and M. C. Allender, University of Illinois, unpubl. data. Consult equipment labels, registered product labels, and the appropriate SDS for regulations on safe and acceptable use.

Agent	Concentration	Contact Time	Application	Efficacy	Human Health and Environmental Risks	Mitigation of Negative Effects
Sodium Hypochlorite[1,2,3,4,5,6,7,8] **(Chlorine Bleach, Clorox™, dry bleach/pool chemical bleach)**	2% solution = 1 cup (226.8 g) dry bleach (56%) per 2 gal (7.6 L) water[7]	2 min	All surfaces, except plastics Dry bleach easier to carry in the field[7] For large equipment, spray and air dry	Ranavirus[1,2,3,4,5] Bd[2,3,5,8] SFD[6] Longer contact times may be more effective for Ranavirus	Negative effects include: Chlorine gas and vapors** Corrosiveness to fabrics, plastics, rubber, and metal Rapid dissipation of disinfectant properties when exposed to air requiring frequent reconstitution to ensure efficacy Test strips available to check viability of bleach solution Toxic to aquatic life	**Handle chlorine in well ventilated area, use dry chemical form, NEVER mix with ammonia-containing products Do not discard solutions in or around water sources unless one of the following dechlorination methods are used[9] Ascorbic acid 1 gram per 100 gal (378.54 L) of water to dechlorinate a 0.1% chlorine solution Sodium ascorbate 2.8 parts per 1 part chlorine Sodium thiosulfate[8,10] 5.6 grams per 10 gal (37.85 L) of 20-ppm chlorine.
	3% = 1:32 dilution of 6% household bleach to water (4 oz bleach per 1 gal water or 118.3 ml to 3.8 L)[1,2,3,4,6]	1 min[2] 2 min[6] 5 min[6]				
	5% = 1.6 cup (380 ml) liquid bleach (6% concentration of sodium hypochlorite) per 2 gal (7.6 L) water[1]	5-15 min				
	200 ppm Formula: [(target conc (ppm)) x (volume of water (gal or L)) x (0.00378)] divided by % active ingredient = mL of bleach needed Example: To make a 200 ppm solution of chlorine bleach in a 5 gal (18.93 L) bucket: [(200ppm)(5 gal)(0.00378)] / 0.06 = 63 mL of chlorine bleach per 5 gal (18.93 L) of water.	10 min				
	10% bleach[5,6]	Scrub & rinse gear with water; submerge gear in solution for 10 min; rinse with water; set in sunlight 1-4 hr; freeze overnight[5] 2 min[6]	Sensitive equipment which cannot be soaked, liberally spray with a 1 part water to 1 part bleach solution[5]			

Agent	Concentration	Contact Time	Application	Efficacy	Human Health and Environmental Risks	Mitigation of Negative Effects
Virkon S™ Virkon Aquatic™ (potassium peroxymonosulfate)[1,2,3,4,8,10,11]	1% solution (1 scoop (1.3 oz or 36.86 g) or 1 tablet per 1 gal or 3.8 L water)[9,11]	1 min[1,2,,3,4] & 5 min[1] 2 min & 10 min[11]	Safe for all field equipment	Ranavirus[1,2,3,4] Bd[2,3,8] SFD[11]	Powder poses risks if inhaled or contacts mucous membranes; must be mixed in a well-ventilated area, preferably outdoors or fume hood Appropriate PPE should be used (splash apron, gloves and safety goggles) Tablets more stable and less hazardous to personnel for transporting and reconstitution Test strips available to check viability of solution	Once in solution non-toxic to amphibians, environment, humans
	0.02%[4]	1 min[4]		Bd[4]		
Phenols (Lysol™)[6]	2-5%	10 min	Hard surfaces Must rinse with water	SFD[6]	Not labeled for use on field gear Irritating to skin	
Ethanol[3,4,6]	70%	10-30 min 1 min[3,4] 2 hr[3]	Surgical equipment; hands; countertops	Ranavirus Bd SFD[6] Bd[3,4] Ranavirus[3,4]	Inactivated by sunlight; evaporates quickly and may not get proper contact time; drying to skin; expensive; not good for field equipment; fixes organics to hard surfaces.	
Benzalkonium Chloride[3,4,6]	0.1% 0.2%	1 min	Surgical equipment	Bd[3,4] SFD[6]		

Agent	Concentration	Contact Time	Application	Efficacy	Human Health and Environmental Risks	Mitigation of Negative Effects
CLR™[6]		10+ min		SFD		
Nolvasan™[1,2,3,4,6] Chlorhexidine	0.75 %	1 min		Ranavirus[1,2,3,4] Bd[2,3] NOT SFD[6]		
Quaternary Ammonium Compounds[6,8] DDAC (e.g., Roccal™, NPD™)	400 ppm 150 ml of DDAC per 50 gallons (189.27 L); to convert for a 5 gallon (18.93 L) sprayer = 15 mL DDAC per 5 gallons (18.93 L) of water	10+ min	Spray or dip for equipment Rinse with water	Bd[8] SFD[6]	DDAC is not allowed to enter a surface water drain or enter water containing fish DDAC is inactivated by the presence of organic matter Quat Chek 1000™ Test Papers to determine viability of solution	Diluted DDAC should be discarded according to State and Federal household chemical standards
	0.16% Roccal™ or NPD™	10+ min				
Heat[3,4,8]	60^0 C	15 min[3] 30 min	Disinfecting equipment, enclosures, footwear and vehicles	Bd[8] Ranavirus		
	Ultraviolet (UV)[3,4]	1 min sterilizing UV		Ranavirus only[3,4]		
	Hot wash[3,4,10] ≥60^0 C	30 min	Cloth bags and equipment, clothing	Bd[8] Ranavirus		