KEEPING THE HEAT ON: WEIGHTED SURVEILLANCE FOR CHYTRID FUNGUS (BATRACHOCHYTRIUM DENDROBATIDIS) IN DIXIE VALLEY TOADS (ANAXYRUS [= BUFO] WILLIAMSI)

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KEEPING THE HEAT ON: WEIGHTED SURVEILLANCE FOR CHYTRID FUNGUS (*BATRACHOCHYTRIUM DENDROBATIDIS*) IN DIXIE VALLEY TOADS (*ANAXYRUS* [= *BUFO*] *WILLIAMSI*)

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ABSTRACT: Introduced fungal pathogens have caused declines and extinctions of naïve wildlife populations across vertebrate classes. Consequences of introduced pathogens to hosts with small ranges might be especially severe because of limited redundancy to rescue populations and lower abundance that may limit the resilience of populations to perturbations like disease introduction. As a complement to biosecurity measures to prevent the spread of pathogens, surveillance programs may enable early detection of pathogens, when management actions to limit the effects of pathogens on naïve hosts might be most beneficial. We analyzed surveillance data for the endangered and narrowly endemic Dixie Valley toad (*Anaxyrus* [= *Bufo*] *williamsi*) from two time periods (2011–2014 and 2019–2021) to estimate the minimum detectable prevalence of the amphibian fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). We assessed if detection efficiency could be improved by using samples from both Dixie Valley toads and co-occurring introduced American bullfrogs (*Lithobates catesbeianus*) and literature-derived surveillance weights. We further evaluated a weighted surveillance design to increase the efficiency of surveillance efforts for *Bd* within the toad's small (<6 km^2) range. We found that monitoring adult and larval American bullfrogs would probably detect *Bd* more efficiently than monitoring Dixie Valley toads alone. Given that no *Bd* was detected, minimum detectable prevalence of *Bd* was <3% in 2011–2014, and <5% (Dixie Valley toads only) and <10% (American bullfrogs only) in 2019–2021. Optimal management for *Bd* depends on the mechanisms underlying its apparent absence from the range of Dixie Valley toads, but a balanced surveillance scheme that includes sampling American bullfrogs to increase the likelihood of detecting *Bd*, and adult Dixie Valley toads to ensure broad spatial coverage where American bullfrogs do not occur, would probably result in efficient surveillance, which might permit timely management of *Bd* if it is detected.

Key words: American bullfrog, amphibian, *Anaxyrus* (= *Bufo*) *williamsi*, *Batrachochytrium dendrobatidis*, chytridiomycosis, conservation, *Lithobates catesbeianus*, weighted surveillance.

INTRODUCTION

Introduced pathogens and emerging wildlife diseases threaten wildlife populations and even entire species. Fungal pathogens have proven to be particularly problematic in the latter 20th and early 21st centuries. White-nose syndrome, caused by the fungus *Pseudogymnoascus destructans*, has decimated some bat species in eastern North America (Blehert et al. 2009; Hoyt et al. 2021), and *Ophidiomyces ophiodiicola*, which causes snake fungal disease, has caused severe lesions and declines of some snake species in the same region (Lorch et al. 2015, 2016). On a larger scale, the amphibian chytrid fungi *Batrachochytrium salamandrivorans* (*Bsal*) and *B. dendrobatidis* (*Bd*) have caused declines of salamanders in Europe (Martel et al. 2013; 2014) and worldwide declines and extinctions of anurans (Stuart et al. 2004; Kilpatrick et al. 2010), respectively.

These pathogens are not uniformly distributed worldwide, however, and early detection of pathogens in naïve populations can be an important component of managing wildlife diseases.

Surveillance programs to detect disease agents provide the option of rapidly responding to introduced pathogens. If management actions to prevent establishment or ameliorate effects of disease are identified, early detection may allow resource managers to act before population-level effects of disease occur. By their nature, such surveillance programs often result in data sets with either no or very few detections (Heisey et al. 2014; Jennelle et al. 2018; Waddle et al. 2020). Interpreting these data to obtain useful information requires nonstandard statistical techniques. For example, Waddle et al. (2020) based their surveillance design for *Bsal* throughout the US on a model of likely susceptibility of salamanders to *Bsal* and routes of entry of the pathogen (Richgels et al. 2016). They further demonstrated how different prior assumptions about occurrence and detection affect posterior inference about whether *Bsal* already occurs in the US (Waddle et al. 2020). Additional efficiency in surveillance studies can be gained by identifying groups that are at higher or lower risk of infection (Heisey et al. 2014; Jennelle et al. 2018) and using weighted surveys to improve the efficiency of surveillance efforts by focusing on segments of the population at greatest risk of disease (Jennelle et al. 2018). Efficient surveillance programs, perhaps including common surrogate species with high risk of infection, might be especially important for rare or endangered species where the consequences of pathogen introduction could be particularly severe.

Dixie Valley toads (*Anaxyrus* [= *Bufo*] *williamsi*; Fig. 1) are found only within the Dixie Valley in the northwestern part of Nevada, USA (39°47′N, 118°4′W; Fig. 2; Forrest et al. 2017; Gordon et al. 2017). This unique toad is restricted to just four spring-fed wetlands across a range of only 6 km^2, where it is threatened by the construction, operation, and expansion of a geothermal plant (Forrest et al. 2017; Gordon et al. 2017; Halstead et al. 2021). In April 2022, the US Fish and Wildlife Service (USFWS) announced the emergency listing of the Dixie Valley toad under the Endangered Species Act, providing immediate federal protections for 240 d (USFWS 2022). The primary cause of concern with regard to the expansion of geothermal energy is the potential for changes to the quantity, temperature, and chemical composition of spring discharge (Huntington et al. 2014). The Dixie Valley toad is also threatened by invasive species, particularly American bullfrogs (*Lithobates catesbeianus*), and by chytridiomycosis caused by *Bd* (Forrest et al. 2013). American bullfrogs are a known vector transmitting *Bd* to more vulnerable native species (Daszak et al. 2004; Garner et al. 2006; Eskew and Todd 2013), and the presence of American bullfrogs in the Dixie Valley increases the likelihood of introduction of *Bd* to Dixie Valley toads. American bullfrogs are abundant in Turley Pond, approximately 10 km south of Dixie Meadows; they are known to co-occur with Dixie Valley toads in one location, Cold Springs Pond (Fig. 2; Forrest et al. 2013). Closely

FIGURE 1. Adult Dixie Valley toad (*Anaxyrus williamsi*) in Dixie Meadows, Churchill County, Nevada, USA. (Photographed by Kris Urquhart, Nevada Department of Wildlife.)

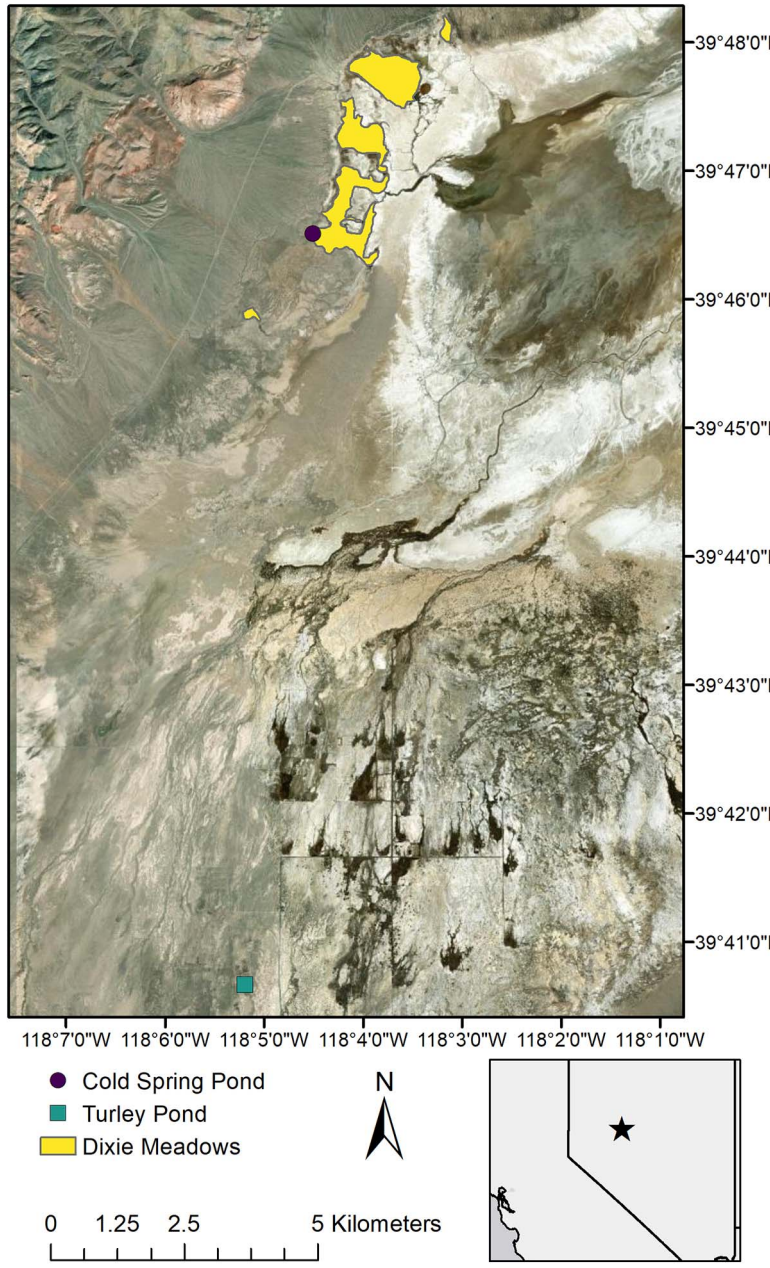

FIGURE 2. Map of Dixie Valley, Nevada, USA, showing locations of sampling sites for Dixie Valley toads (*Anaxyrus williamsi*) and American bullfrogs (*Lithobates catesbeianus*), 2011–2021.

related western toads (*Anaxyrus boreas*) in the Rocky Mountains and Yosemite toads (*Anaxyrus canorus*) in the Sierra Nevada are highly susceptible to *Bd* (Muths et al. 2003; Pilliod et al. 2010; Lindauer and Voyles 2019); therefore it is considered that introduction of *Bd* to the Dixie Valley toad population could be catastrophic.

The objectives of our study were twofold. First, we sought to estimate the minimum detectable prevalence of *Bd* in Dixie Valley toads. Second, we evaluated the efficiency of

TABLE 1. *Batrachochytrium dendrobatidis* sampling effort by species (Dixie Valley toad, *Anaxyrus williamsi*, or American bullfrog, *Lithobates catesbeianus*) and location in the Dixie Valley, Nevada, USA, 2011–2021. We obtained all samples between April and June each year.

Year	Site	Species	Individuals swabbed
2011	Dixie Meadows (north)	Dixie Valley toad	39
	Turley Pond	American bullfrog	11
2012	Dixie Meadows (north)	Dixie Valley toad	53
	Turley Pond	American bullfrog	32
2014	Dixie Meadows (Cold Spring area)	Dixie Valley toad	35
2019	Dixie Meadows (throughout)	Dixie Valley toad	47
	Dixie Meadows (Cold Spring area)	American bullfrog	7
2020	Dixie Meadows (throughout)	Dixie Valley toad	14
2021	Dixie Meadows (throughout)	Dixie Valley toad	40
	Dixie Meadows (Cold Spring area)	American bullfrog	10

different weighted surveillance protocols for detecting a minimum specified *Bd* prevalence in Dixie Valley toads at Dixie Meadows at a specified degree of certainty using different potential surveillance groups.

MATERIALS AND METHODS

Field methods

We sampled amphibians in the Dixie Valley for *Bd* on several occasions during two time periods, the first time being 2011, 2012, and 2014 and the second 2019–2021. We visually surveyed for amphibians and captured as many individuals as possible by hand, using a new pair of disposable nitrile (2011–2014; Dynarex Corporation, Orangeburg, New York, USA) or prerinsed, powderless, vinyl gloves (2019–2021; Gorilla Supply, Elk Grove Village, Illinois, USA) to handle each animal. In 2011–2014, we used a Sterile Omni Swab (Whatman [Cytiva], Little Chalfont, UK) to sample skin cells from each animal's venter, flanks, and groin. We swabbed each amphibian a total of 25 times using the applicator, which was then ejected into a 2-mL sterile tube filled with a buffer solution containing 70% ethanol and stored at 4 C until processing. In 2019–2021, we used Sterile Medical Wire swabs (MW-113, Medical Wire & Equipment, Corsham, UK), and we swabbed each amphibian a total of 25 times (five swipes on the webbing of each rear foot, ventral surface of each thigh, and the animal's venter). We developed this protocol based on Puschendorf and Bolaños (2006) and Van Rooij et al. (2011) to ensure different body locations were swabbed thoroughly to pick up *Bd* DNA. Swabs were stored in 1.5-mL tubes each containing 20 µL of sterile deionized water or sterile phosphate-buffered saline. Surveillance effort and sample sizes for Dixie Valley toads and American bullfrogs varied across years (Table 1). All sampling gear was thoroughly decontaminated using a 10% bleach solution with 15 min contact time at each site immediately after sampling to prevent spreading *Bd* and other invasive species or pathogens between sites. To minimize stress, animals were processed immediately and were released at the point of capture. No individuals showed obvious signs of distress during sampling, and all Dixie Valley toads swam or hopped away immediately upon release.

Laboratory methods

All 2011, 2012, and 2014 samples were assayed within 1 mo of being collected for the presence of *Bd* by a commercial laboratory (Pisces Molecular, Boulder, Colorado, USA) as described (Annis et al. 2004) with modifications to increase sensitivity and specificity: the use of hot start Taq polymerase, increasing annealing temperature from 60 C to 65 C, increasing anneal segment time from 45 s to 105 s, and increasing the number of cycles from 30 to 45 (J. Wood pers. comm.). In 2012, we used skin swabs from *L. catesbeianus* to assay for *Bd* infection intensities using quantitative real-time PCR (qPCR). We extracted DNA from swabs by centrifuge (~16,000 × G for 3 min), resuspended the pellets in lysis buffer,

added carrier DNA (10 μg salmon sperm DNA), and isolated DNA using spin-column purification (Qiagen DNeasy Blood and Tissue Kit; Qiagen, Valencia, California, USA). Prepared DNAs were assayed for the presence of the *Bd* ribosomal RNA internal transcribed spacer (ITS) region following the methods of Annis et al. (2004), modified as indicated previously. Amplifications were conducted for 45 cycles on a Stratagene MX4000 Multiplex Quantitative PCR Cycler (Agilent Technologies, Santa Clara, California, USA), with standard curves developed for each reaction by inclusion of serial 10-fold dilutions of linearized plasmid DNA containing the *Bd* ribosomal RNA region.

For samples collected in 2019–2021, we extracted DNA from swabs as described by Hyatt et al. (2007), except that 125 μL of PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California, USA) and 100 mg of zirconium/silica lysis beads (Biospec Products, Bartlesville, Oklahoma, USA) were used so that the entire swab was immersed. The bead-beating steps were conducted using a FastPrep®-24 homogenizer (MP Biomedicals, Santa Ana, California, USA). We used a real-time TaqMan PCR for detection of *Bd* on the extracted DNA as described (Blooi et al. 2013, 2016). We ran reactions on the 7,500 fast real-time (RT) PCR system (Applied Biosystems) using QuantiFast Probe RT-PCR mastermix kit with ROX dye (Qiagen) and BSA as per the kit instructions. We used 5 μL of the PrepMan® solution containing the extracted DNA as template for the PCR. We included a negative extraction control and a standard curve run in duplicate on each PCR plate. The standard curve consisted of five different concentrations of the target sequence for *Bd* inserted into plasmids. The concentrations of the standards occurred at 10-fold dilutions ranging from 110 to 1,100,000 copies (0.5–5,000 fg DNA) per reaction (on some initial runs, the standard range was 11–110,000 copies per reaction). The threshold for signal detection was set at 5% of the maximum fluorescence of the standards run for that assay. We considered a positive detection of *Bd* DNA if a detectable signal existed at 37 or fewer PCR cycles and no detection in all other cases. We calculated the efficiency of each run using standard curve amplification and repeated PCR plates with an efficiency of less than 90% or greater than 110%. Data for 2019–2021 are available in ScienceBase (Kleeman et al. 2021).

Analytical methods

We estimated the minimum detectable prevalence of *Bd* in Dixie Valley toads at Dixie Meadows using a weighted surveillance approach. The weights refer to the value of sampling different classes of individuals with different relative risks of infection (Heisey et al. 2014; Jennelle et al. 2018). The concept of using surveillance weights is indifferent to the mechanisms that generate differential infection risks, but rather relies on robust estimates of infection risk from existing data. We estimated surveillance weights and minimum detectable prevalence of *Bd* in two steps. First, we estimated surveillance weights focusing on four classes of *Bd* host (adult American bullfrogs, larval American bullfrogs, adult western toads, and larval western toads) using data from Richardson et al. (2014) on prevalence of endemic *Bd* in these and other species of amphibians. We used western toads as a surrogate for Dixie Valley toads because Dixie Valley toads were formerly considered western toads and western toads are the most closely related species for which data were available. We chose to use these data to estimate surveillance weights because Richardson et al. (2014) sampled *Bd* from multiple amphibian host classes (species and life stages) across the same time and space where *Bd* occurrence was widespread. We applied a discrete proportional hazards model (Heisey et al. 2014; Jennelle et al. 2018) to estimate the baseline *Bd* detection rate (i.e., apparent prevalence) in adult western toads and the relative *Bd* detection rate among I additional amphibian classes defined by species and life stage using a Bernoulli likelihood,

$$L(\pi_{I,j}|y_{i,I,j}) = \pi_{I,j} * (1 - \pi_{I,j}),$$

where $y_{i,I,j}$ was the observed detection or nondetection of the ith individual, belonging to surveillance class I, sampled from spatial unit j, with probability of detection $\pi_{I,j}$. Probability of detection was defined by the proportional hazards model and mapped onto the probability scale using the inverse of complementary log-log link (cloglog) function (Heisey et al. 2014):

$$\pi_{I,j} = 1 - \exp(-\exp([\mu_{\text{ref}} + x_I\beta + a_j]),$$

where μ_{ref} was an intercept term that represented the reference class (adult western toads) against which the other surveillance classes were compared, x_I was an indicator variable for the surveillance class

that each sampled amphibian i belonged to, β was a vector of coefficients that estimated the surveillance class log infection rate ratios, and a was a random effect for spatial unit j. We applied the watershed designation from Richardson et al. (2014) for each sample as the sample unit to account for spatial variation in detection rate. We used a Bayesian approach with Gibbs sampling implemented in JAGS (Plummer 2017) to estimate the relative surveillance weights using uninformative priors, *norm*(0, 1000), on the cloglog scale for the parameters for reference (μ_{ref}) and surveillance class coefficients (β). We used an uninformative uniform prior, unif(0, 100), on the standard deviation for the spatial unit random effect, a. Model specification, fitting, and parameter estimate details are presented in Supplementary Materials Table S1.

Second, we calculated minimum detectable prevalence of *Bd* from samples collected in the Dixie Valley from two time periods (2011–2014 and 2019–2021; Table 1). We used the surveillance weights estimated for adult American bullfrogs calculated above as closed-form informative priors for the surveillance value of adult American bullfrogs relative to adult Dixie Valley toads. We incorporated diagnostic test sensitivity to make inference to true prevalence using a similar likelihood and proportional hazards model with cloglog link function:

$$L(\pi_I|\mathrm{Se}, y_{i,I}) = (\pi_I * \mathrm{Se}) * [\pi_{I,j} * (1 - \mathrm{Se}) + (1 - \pi_I)],$$

$$\pi_I = 1 - \exp(-\exp([\mu_{\mathrm{ref}} + x_I\beta]).$$

The informative priors for surveillance weights were applied as the normal distribution mean and standard deviation for $\beta_{\mathrm{bullfrog}}$ and we used uninformative priors, norm(0, 1,000), on the cloglog scale for the reference prevalence (μ_{ref}). Repeat sampling was not performed to be able to estimate diagnostic sensitivity directly, so we used a laboratory-derived sensitivity prior of Beta(100, 5) to represent high probability of detection of *Bd* in a sample with approximately 100 copies of the PCR target intergenic transcribed spacer (ITS) sequence. Model specification, fitting, and parameter estimate details are presented in the Supplementary Materials.

To develop an efficient survey protocol, we simulated the impact of sampling different numbers of toads and bullfrogs with different weights to prescribe the appropriate sample size of the different classes of *Bd* hosts to ensure 95% certainty of detecting a minimum specified prevalence. We simulated scenarios to illustrate the effectiveness of several sampling designs using the calculations in Jennelle et al. (2018):

$$n_{\mathrm{survClass}} = \frac{E_{\mathrm{adj}-(n_{\mathrm{ref}} * W_{\mathrm{survClass}})}}{W_{\mathrm{ref}}},$$

$$E_{\mathrm{adj}} = \frac{\ln(1 - \mathrm{conf})}{\ln(1 - \pi * \mathrm{Se})},$$

where $n_{\mathrm{survClass}}$ is the sample size of a nonreference surveillance class (adult bullfrogs), n_{ref} is the sample size of the reference surveillance class (adult toads), $W_{\mathrm{survClass}}$ is the surveillance weight estimated for the nonreference surveillance class, W_{ref} is the surveillance weight of the reference surveillance class (fixed to 1), and E_{adj} is a constant that accounts for the desired confidence of detection (conf = 0.95) for a given prevalence (π = 0.01, 0.025, 0.05, or 0.10) with a given diagnostic test with sensitivity, Se (probability of correctly detecting an infected individual). We chose 95% to illustrate this example, but surveillance design should consider sensitivity estimates for the methods being used for a specific application.

RESULTS

The likelihood of detecting *Bd* at a site where it occurred varied among species and life stages. Surveillance weights calculated from the data presented in Richardson et al. (2014) indicated that detection of *Bd* in larval western toads was only 0.04 (0.01–0.11) times as likely as in adult western toads for the same number of individuals sampled (Table 2). In contrast, detection of *Bd* was 1.69 (0.96–2.93) times more likely in adult American bullfrogs than adult western toads and 10.2 (4.62–22.9) times more likely in larval American bullfrogs than adult western toads (Table 2).

None of the amphibians we sampled in Dixie Meadows tested positive for *Bd*. Estimated minimum detectable prevalence of *Bd* in Dixie Meadows was 0.8% (<0.01–2.9%) for adult Dixie Valley toads sampled in 2011–2014. Using the weighted surveillance model for Dixie

TABLE 2. Surveillance weights estimated from relative detection rates of *Batrachochytrium dendrobatidis (Bd)* for adult and larval American bullfrogs (*Lithobates catesbeianus*) and larval western toads (*Anaxyrus boreas*), relative to adult western toads from detection reported by Richardson et al. (2014). β (SE) = coefficient from complementary log-log regression model and its standard error; *w* [95% CI] = surveillance weight and its 95% confidence interval.

Host class	β (SE)	*w* [95% CI]
Adult toads (reference class)	–	1[a]
Larval toads	−3.27 (0.60)	0.04 [0.01–0.11]
Adult bullfrogs	0.52 (0.28)	1.69 [0.96–2.93]
Larval bullfrogs	2.32 (0.40)	10.16 [4.62–22.9]

[a] Fixed at 1 as the reference class.

Valley toads and American bullfrogs sampled in 2019–2021, the minimum detectable prevalence was 0.6% (<0.01–1.9%) and 2.1% (<0.01–7.4%), respectively. Simulations to estimate sample sizes necessary to detect *Bd* indicated that the sampling in both 2011–2014 and 2019–2021 were adequate to detect *Bd* in Dixie Valley toads if target detectable prevalence was <5% (Fig. 3A). Sampling of American bullfrogs, however, was only sufficient to meet a target detection prevalence of <10% (Fig. 3B). Although we did not detect *Bd* in Dixie Meadows, *Bd* prevalence among American bullfrogs at Turley Pond was 18% in 2011 and 75% in 2012. Combining samples of toads and American bullfrogs might increase efficiency of detecting *Bd* in Dixie Meadows if both species are equally available for sampling (Fig. 3).

DISCUSSION

Although none of the amphibians we tested at Dixie Meadows were positive for *Bd*, inference about the prevalence of *Bd* at Dixie Meadows differed among species because of differences in sampling effort and expected prevalence of *Bd*. We were able to estimate lower minimum detectable prevalence for Dixie Valley toads than for American bullfrogs despite expected relative surveillance weight of the latter being higher. The greater certainty about absence of *Bd* in Dixie Meadows provided by Dixie Valley toads was because

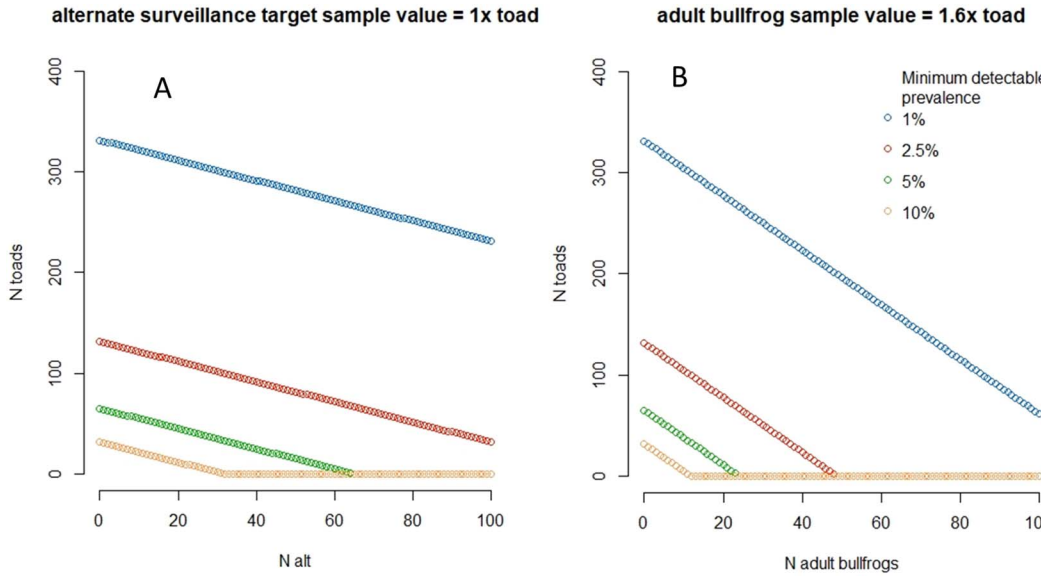

FIGURE 3. Number of individuals required to be sampled to achieve 95% confidence that prevalence is below a given threshold for two classes of surveillance targets with an estimated test sensitivity of 95%. (A) Combination of samples if the two classes have equal surveillance value. (B) Combination of samples if the second surveillance class has 1.6× the surveillance value of the reference surveillance class.

we sampled many more toads than bullfrogs. All else being equal, however, we expect that surveillance of adult bullfrogs would allow detection of *Bd* at Dixie Meadows with approximately 41% fewer individuals sampled. Dixie Valley toads are more abundant than American bullfrogs at Dixie Meadows, and sampling Dixie Valley toads for *Bd* is often incidental to other monitoring activities for the species, whereas sampling bullfrogs for *Bd* was typically the sole purpose for capturing bullfrogs. Thus, whether sampling adult bullfrogs results in a more efficient surveillance protocol depends on the relative effort required to capture bullfrogs and toads.

In contrast to the relatively small difference in surveillance weights between adult toads and bullfrogs, larval bullfrogs had much higher surveillance weights. Indeed, if relative prevalence (i.e., risk of infection) of *Bd* among taxa in our study area is similar to that of Richardson et al. (2014), then sampling a single larval American bullfrog would be equivalent to sampling about 10 adult Dixie Valley toads. Sampling bullfrog larvae for *Bd* could have the benefit of removing individuals of an invasive species while increasing the probability of detecting *Bd* in Dixie Meadows. The estimate of surveillance weights depends, however, on the amount of auxiliary information available to inform those estimates. Because of the small sample size of American bullfrog larvae in Richardson et al. (2014), the high surveillance value also comes with large uncertainty (Table 2).

Detection and prevalence of *Bd* in American bullfrogs at Turley Pond is important because *Bd* at that site poses a constant threat of *Bd* introduction into Dixie Meadows, despite the lack of detections in Dixie Meadows to date. American bullfrogs pose a double threat to Dixie Valley toads both as an invasive species (Miaud et al. 2016) and because they act as a vector for *Bd* (Daszak et al. 2004; Garner et al. 2006; Eskew and Todd 2013; Yap et al. 2018). We propose two nonexclusive hypotheses to explain the lack of *Bd* in Dixie Meadows that have very different implications for management and surveillance efforts: 1) *Bd* has not been introduced to Dixie Meadows; 2) *Bd* is not able to establish in Dixie Meadows because of 2a) water temperature and/or 2b) water chemistry in the unique geothermal environment of Dixie Meadows.

First, despite occurring in American bullfrogs at high prevalence in nearby Turley Pond (Fig. 2), it is possible that *Bd* has not yet been introduced to Dixie Meadows, although it is only 10 km distant. Researchers at Dixie Meadows take great care to avoid introducing *Bd* or other diseases to the site, but other mechanisms of transport probably exist. For example, other visitors to Dixie Meadows might be less aware of the threat *Bd* poses to amphibians and unknowingly transport the pathogen on clothing or equipment. In addition to human visitors, cattle graze much of the Dixie Valley and frequent Dixie Meadows. They or other animals might serve as mechanical vectors moving *Bd* from Turley Pond or other sources to Dixie Meadows. The precise mechanisms by which *Bd* disperses remain unclear, and it is also unclear how long *Bd* has been present in Turley Pond. Thus, although we cannot rule out the hypothesis that *Bd* has not yet been introduced to Dixie Meadows, introduction of the pathogen to Dixie Meadows remains a constant threat.

Second, the unique geothermal habitat may provide a refuge from *Bd* to Dixie Valley amphibians. The prevalence of *Bd* and the severity of chytridiomycosis are particularly influenced by temperature (Woodhams et al. 2008). Field studies from across the globe show *Bd* infections are generally more severe in winter months and when hosts are found in lower temperatures (Bradley et al. 2002; Berger et al. 2004; Murray et al. 2009; Voordouw et al. 2010). Water temperatures at Turley Pond at the time of sampling American bullfrogs for *Bd* were 21–24 C, whereas we have measured water temperatures at Dixie Meadows as high as 80 C (Kleeman and Halstead 2022). Growth of *Bd* ceases at temperatures >28 C (Johnson and Speare 2003; Piotrowski et al. 2004), and short-term exposure to elevated temperatures (27–37 C)

cleared *Bd* infections from five amphibian species (Woodhams et al. 2003; Berger et al. 2004; Retallick and Miera 2007; Chatfield and Richards-Zawacki 2011). Amphibians may also be less susceptible to *Bd* when they experience constant higher temperatures, because of increased effectiveness of their immune responses (Andre et al. 2008; Murphy et al. 2011; Raffel et al. 2013). Repeated exposures to *Bd* followed by clearance induced by temperatures of 30 C can confer immunological resistance to the pathogen (McMahon et al. 2014).

Geothermal springs may provide amphibians with refugia from *Bd* (Schlaepfer et al. 2007; Forrest and Schlaepfer 2011). Most permanent sources of water for Dixie Valley toad breeding habitat are geothermal springs with source temperatures high enough to clear *Bd* infections from amphibians. In addition to the benefits and protection that warm water may provide, water chemistry may also play a role in protecting amphibians from *Bd* and chytridiomycosis. Within the Greater Yellowstone Ecosystem, western toads breed predominantly in geothermal ecosystems (Klaver et al. 2013), which also appear to protect them from redleg syndrome or other sources of mortality (Carey 2000; Hawk 2000). Salt (NaCl) concentrations greater than 2 ppt significantly reduce host *Bd* infection loads (Stockwell et al. 2015), suggesting that warm, saline wetlands may provide refuges from chytridiomycosis (Heard et al. 2014). The salts in the Humboldt Salt Marsh adjacent to Dixie Meadows are primarily NaCl (Garcia et al. 2015), so it is possible that the combination of heat and water chemistry present within Dixie Valley toad habitat is providing amphibians with refuge from *Bd* and chytridiomycosis.

The close affiliation of Dixie Valley toads with aquatic environments, and sensitivity to water temperature (Halstead et al. 2019, 2021), make them vulnerable to changes in the aquatic environment. Geothermal energy development in California and Nevada has resulted in both increases and decreases to spring discharges (including complete drying of geysers and springs), heating and cooling of spring discharges, and land subsidence (Sorey 2000), and therefore presents a substantial threat to the species (Forrest et al. 2017; Gordon et al. 2017). Brumation by Dixie Valley toads in warmer water near hot springs suggests that the toads select overwintering sites where the water temperature is likely to remain stable, and that alteration of historical patterns in the amount of water coming from springs or water temperature during brumation may be lethal (Halstead et al. 2021). Furthermore, overwintering may be a critical bottleneck for temperate amphibians that are infected with *Bd*, because amphibian immune system responses are suppressed by low temperatures (Wetsch et al. 2022). Maintaining thermally suitable surface water yearround throughout the highly restricted range of Dixie Valley toads is essential for ensuring their persistence in this desert ecosystem.

The mechanism(s) by which Dixie Meadows has remained *Bd* free (or with very low *Bd* prevalence) have very important implications for management. If *Bd* is not present because it has not been introduced, continued surveillance for *Bd* and other novel, lethal pathogens is important to enable early intervention. To our knowledge, no rapid response plan exists for Dixie Valley toads should *Bd* be detected in Dixie Meadows, but management of *Bd* generally consists of controlling the spread of *Bd*, establishing assurance colonies, and preventing or treating chytridiomycosis (Woodhams et al. 2011; Cook et al. 2022; Knapp et al. 2022). The lack of additional Dixie Valley toad populations means that the consequences of epidemic infection could be severe; careful monitoring and surveillance would be needed to enable early action to prevent declines and potential extinction of the species. If *Bd* is not present because the thermal and chemical environment is unsuitable for the pathogen, then continued surveillance and management resources put into preparation for a potential *Bd* outbreak may be suboptimal. Weighted surveillance offers the benefit of increasing the probability of

detecting *Bd* in Dixie Meadows by sampling alternative hosts that are likely to have higher prevalence of the pathogen. Surveying larval American bullfrogs, in particular, could offer an approximate 10-fold increase (per sampled individual) in the potential early detection of *Bd* relative to sampling adult Dixie Valley toads. The increased sampling efficiency of monitoring larval bullfrogs, however, is limited because bullfrogs only occur in Cold Spring Pond, a very small portion of the range of Dixie Valley toads. We suggest that a balanced surveillance scheme including sampling larval bullfrogs in Cold Spring Pond and adult Dixie Valley toads throughout Dixie Meadows will probably offer the best opportunity for early detection of *Bd* and permit a management response if and when *Bd* is found.

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SUPPLEMENTARY MATERIAL

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