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Dynamics of Amphibian Pathogen Detection Using Extended Museum Specimens

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ABSTRACT: Natural history collections have long served as the foundation for understanding our planet's biodiversity, yet they remain a largely untapped resource for wildlife disease studies. Extended specimens include multiple data types and specimen preparations that capture the phenotype and genotype of an organism and its symbionts—but preserved tissues may not always be optimized for downstream detection of various pathogens. Frogs are infected by an array of pathogens including *Batrachochytrium dendrobatidis* (Bd), *Ranavirus* (Rv), and Amphibian Perkinsea (Pr), which provides the opportunity to study differences in detection dynamics across tissue types. We used quantitative PCR protocols to screen two tissue types commonly deposited in museum collections, toe clips and liver, from two closely related host species, *Rana catesbeiana* and *Rana clamitans*. We compared Bd, Rv, and Pr infection prevalence and intensity between species and tissue types and found no significant difference in prevalence between species, but Bd intensity was higher in *R. clamitans* than *R. catesbeiana*. Toe tissue exhibited significantly higher Bd infection loads and was more useful than liver for detecting Bd infections. In contrast, Rv was detected from more liver than toe tissues, but the difference was not statistically significant. Our results support the use of extended specimen collections in amphibian disease studies and demonstrate that broader tissue sampling at the time of specimen preparation can maximize their utility for downstream multipathogen detection.

Key words: Amphibian Perkinsea, amphibians, *Batrachochytrium dendrobatidis*, Chytridiomycosis, frogs, holistic specimen, natural history collections, *Ranavirus*.

Biospecimen repositories hold an essential role in our understanding of global biodiversity. Extended specimens provide broad specimen preparation and data types that document the physical and molecular components of organisms and their communities (Webster 2017). The expansion of specimen-associated genomic resources has also been critical in the study of wildlife diseases, with recent emphasis on zoonotic pathogens (Colella et al. 2021). Research collections enable multiple facets of pathogen research, such as identifying host species (e.g., Yates et al. 2002) and establishing the timeline of pathogen introduction into novel communities (e.g., Karwacki et al. 2021). Although some studies are designed to sample focal species or specific tissues associated with their targeted pathogen, specimen-driven disease research relies on the condition of samples and data previously collected for other purposes. Extended specimens broaden the utility of museum collections, but preparing and preserving valuable material requires time, effort, and funding, which are often limited resources. An evaluation of which preservation techniques maximize pathogen detection in multiple taxa is needed to help direct collection-building efforts.

Amphibians have experienced major species-level declines over the last century, largely attributed to the spread of novel pathogens and parasites (Fisher and Garner 2020). *Batrachochytrium dendrobatidis* (Bd) is a well-studied fungal pathogen that affects the keratinized layer of amphibian skin, causing the amphibian disease chytridiomycosis. This disease has been linked with localized extirpations, widespread population declines, and extinctions on a global scale (Fisher and Garner 2020). A second group of pathogens, generalist lineages in the genus *Ranavirus* (Rv), enter the bloodstream of a host causing edema, lesions, and tissue necrosis in amphibians (Brunner et al. 2021). These pathogens are known to infect >170 ectothermic vertebrates, including 105 amphibian species (Brunner et al. 2021). A third pathogen, referred to as amphibian Perkinsea (Pr), is a protozoan parasite that has been linked to mortality events in frog populations across the US since the 1990s (Isidoro-Ayza et al. 2017). Since its discovery, severe Perkinsea infection has become the third most

common infectious disease known to cause mortalities in wild frog communities (Isidoro-Ayza et al. 2017). This organism has been found mainly in tadpoles, where it causes histopathologic lesions in major organs, but little is understood regarding its infection dynamics across life stages or host species.

Different amphibian pathogens have diverse mechanisms of infection and sites of replication. Therefore, the World Organisation for Animal Health (WOAH, previously OIE) provides different recommendations for detection. *The Manual of Diagnostic Tests for Aquatic Animals* instructs that DNA extracted from skin or skin swabs should be used to detect Bd infections (WOAH 2021a), whereas DNA extracted from affected organs such as liver, kidney, spleen, lung, and skin tissue are recommended for Rv detection (WOAH 2021b). Detection and infection load accuracy of Bd and Rv have been compared across sampling methods, including the site of tissue sample taken (Puschendorf and Bolaños 2006; Ford et al. 2022); timing of tissue collection postexposure (Greer and Collins 2007; Ford et al. 2022); the extent of pathogen exposure (Ford et al. 2022); and which tissue types should be used to detect and quantify targeted infection loads accurately (Clare et al. 2016; Ford et al. 2022). Each choice influences downstream detection of nontarget pathogens, but cannot always be controlled or predicted when studying natural populations. Furthermore, retroactively detecting infections using specimen tissues after formalin fixation has proven unreliable (Adams et al. 2015), making the subsampling of tissues prior to preservation strongly recommended (Zimkus and Ford 2014). It is therefore crucial to determine how to maximize pathogen detection and minimize the destructive sampling required for current molecular screening techniques. In this study, we assessed differences in quantitative PCR (qPCR) detection of three amphibian pathogens (Bd, Rv, and Pr) between two tissue types commonly deposited in amphibian frozen tissue repositories.

We selected toe clip and liver samples representing 102 individuals of two widespread host species, the American bullfrog (*Rana* (*Lithobates*) *catesbeiana*; $n=53$) and green frog (*Rana* (*Lithobates*) *clamitans*; $n=49$), for a total of 204 tissues. Individuals were collected under approved state permits from May to July 2022 along roadways and lentic systems across much of the central and eastern US (Fig. 1; Supplementary Material Table S1). Frogs were either salvaged as roadkill or caught by hand, euthanized following animal care protocols (20-201006-MC) approved by the University of New Mexico Institutional Animal Care and Use Committee, and dissected as soon as possible after initial encounter. Toe and liver tissues of the same individual were sampled with tools sterilized before and after every individual, but not between tissue types. Tissues from the same individual were flash frozen in liquid nitrogen in the same cryovial, then transported to the Museum of Southwestern Biology, Albuquerque, New Mexico, US, for deposition. Our sampling procedures were designed to maximize pathogen detection within an individual, regardless of the potential for cross-contamination between tissue types. Cryovials were then transferred to the molecular lab and stored in −20 C freezers until time of extraction. Toe and liver tissues were separated, and DNA was extracted from each tissue type using an E.Z.N.A. Tissue DNA Extraction Kit (Omega Bio-tek, Inc., Norcross, Georgia, USA) following the manufacturer's protocol. The DNA concentrations were measured using a Broad Range Qubit Assay (Invitrogen, Eugene, Oregon, USA) and standardized to a concentration between 9 and 15 ng of DNA per microliter. Extractions were then screened in duplicate with positive controls (5 µL at 10 ng/µL concentration of known positive samples from the Arctos museum database (Bd: MSB:Herp:104601; Rv: MSB:Herp:104600; Pr: MSB:Herp:104643; Arctos Community 2024; Cicero et al. 2024; see Supplementary Material Table S1) and negative controls (molecular grade water) for pathogen presence using established qPCR protocols (Bd: Boyle et al. 2004; Rv: Allender et al. 2013; Pr: Karwacki et al. 2018).

Samples were initially screened in two independent runs for each pathogen type. Samples that tested positive for the target pathogen were then screened twice more, for

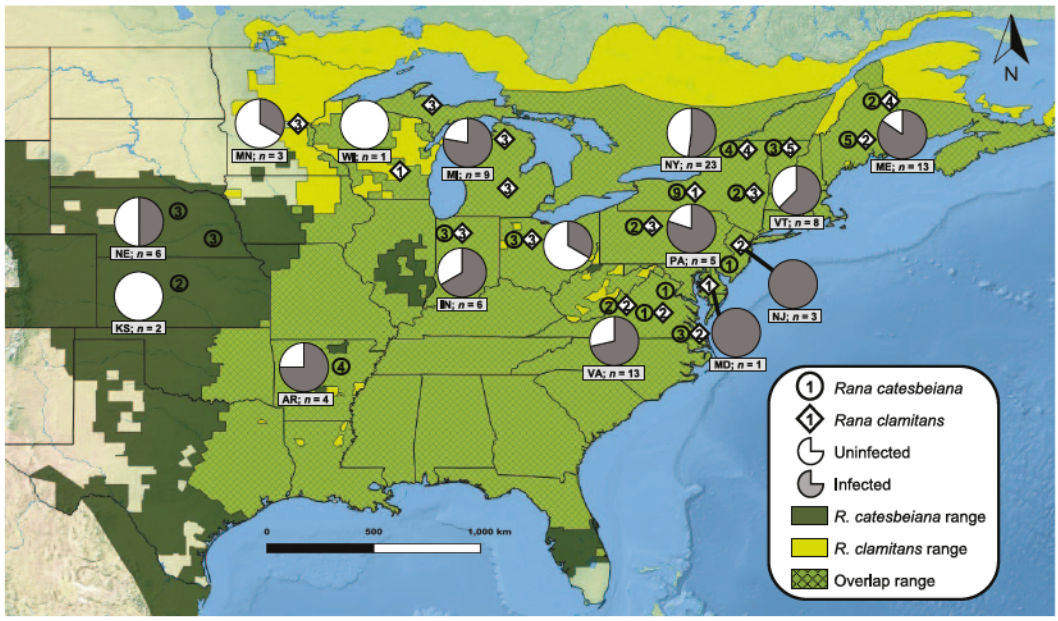

FIGURE 1. Map of sampling distribution across the ranges of the American bullfrog (*Rana catesbeiana*) and green frog (*Rana clamitans*) where numbers within shapes represent the count of individuals of each species collected from one locality. Pie charts denote the collective *Batrachochytrium dendrobatidis* (Bd) and ranavirus (Rv) infection ratio for both species for each state with sample sizes below. Range maps are from the International Union for the Conservation of Nature (IUCN SSC Amphibian Specialist Group 2022a, b).

a total of at least four independent qPCR runs per sample. Infection intensity was calculated using cycle threshold (Ct) values generated from qPCR amplification curves converted into starting quantities (SQ) based on the standard curve run on every qPCR plate. To account for differences in reagent sensitivity between batches of qPCR, SQ values for positive samples were averaged (mean) across the two final runs. We summarized infection occurrences and intensities for each host species and pathogen type and conducted statistical analyses in R (R Core Team 2023). R code and data sets are available from figshare (Barrow et al. 2024). Differences in infection prevalence for each pathogen were assessed between species using Pearson's chi-squared tests. We tested for differences in detection between tissue types within the same individual using McNemar chi-squared tests for paired samples. We log-transformed the SQ values to normalize distributions of infection intensity. Infection intensities were then compared for each pathogen between species using unpaired two-sample t-tests. For individuals that tested positive using both liver and toe tissues, we tested for differences in infection intensity based on tissue type using paired two-sample *t*-tests. For this analysis, we pooled the data for *R. clamitans* and *R. catesbeiana* to increase sample size.

We found that 64% (65/102) of individuals were infected with at least one pathogen (Fig. 1). For both species, we found the highest prevalence of Bd, with 55% of individuals infected (51% of *R. catesbeiana* and 59% of *R. clamitans*), followed by Rv with 21% of individuals infected (25% of *R. catesbeiana* and 16% of *R. clamitans*); we found no cases of Pr (Table 1). We found no deviation in the expected infection prevalence between the two species for Bd (χ^2=0.405; P=0.525) or Rv (χ^2=0.606; P=0.436). When comparing infection intensity, we found that *R. clamitans* had slightly higher Bd SQ values than *R. catesbeiana* (t=−2.286; P=0.0266; Fig. 2A), but there was no significant difference in Rv SQ values between species (t=0.421; P=0.68; Fig. 2B).

TABLE 1. Count of individual amphibian quantitative PCR screening results for *Batrachochytrium dendrobatidis* (Bd), ranavirus (Rv), and amphibian Perkinsea (Pr) across two frog species, the American bullfrog (*Rana catesbeiana*) and green frog (*Rana clamitans*), and two tissue types (toe clip and liver). Consistent positive and negative counts are individuals with both liver and toe tissues agreeing. Cohen's kappa values show minimal agreement (<0.4) between the two tissue types screened for each pathogen.

Species binomial	Pathogen type	Percent positive	Consistent positive	Consistent negative	Liver only	Toe only	Cohen's kappa (κ)
Rana catesbeiana n=53	Bd	51%	3	26	0	24	0.109
	Rv	25%	4	40	6	3	0.373
	Pr	0%	0	53	0	0	NA[a]
Rana clamitans n=49	Bd	59%	8	20	1	20	0.214
	Rv	16%	2	41	4	2	0.335
	Pr	0%	0	49	0	0	NA[a]

[a] NA = not applicable.

When comparing tissue types, 98% of Bd-positive individuals were detected from toe tissue samples compared to 21% detected from liver tissues (Table 1). Detection significantly differed between toe and liver samples for each species analyzed separately (*R. catesbeiana*: McNemar's χ^2=22.042; P<0.0001; *R. clamitans*: McNemar's χ^2=15.429; P<0.0001) and when combined (McNemar's χ^2=39.2; P<0.0001). In contrast, Rv detection did not differ significantly between tissue types for either species separately or when combined (McNemar's χ^2=1.067; P= 0.3017). Of the 21 Rv-positive individuals, six tested positive from both tissue types, 10 tested positive from liver only, and five tested positive from toe only (Table 1). We found higher Bd infection intensity in toe tissues compared to liver tissues that tested positive within the same individual (paired t-test: t=−8.038; P=0.0013; Fig. 2C). There was no difference in Rv infection intensity between tissue types for individuals with consistent positives (paired t-test: t=−1.345, P=0.236; Fig. 2D), but we note that sample sizes were low.

Our study highlights the different detection rates and infection intensities for two major amphibian pathogens depending on the tissue type screened, emphasizing the importance of incorporating extended specimens into wildlife pathogen research. One important caveat about our study design is the potential for cross-contamination between tissue types within an individual, because tissues were sampled with the same tools and stored temporarily in the same cryovials. Interestingly, our results show that this procedure did not necessarily lead to the detection of positive infections from both tissues; that is, many individuals only tested positive based on one tissue type. Given the nature of Bd infecting keratinized epithelial cells, it is unsurprising that our findings support toe (including skin) tissues to diagnose samples for Bd (Marantelli et al. 2004). In contrast, for Rv infection, our results demonstrate increased detection by using both tissue types. These findings largely agree with previously suggested diagnostic flexibility, because Rv presents in both internal and external tissues in the host (Duffus et al. 2015). The lower prevalence of Rv than of Bd in both species is consistent with previous work that suggested postmetamorphic green frogs may not carry sufficient pathogen loads to determine effective sampling strategies (Forzán and Wood 2013). We also did not detect any Pr in our study, which supports the hypothesis that amphibian Perkinsea infections are comparatively rare and localized when found in postmetamorphic stages (Isidoro-Ayza et al. 2017). Future studies should include collecting tissues and vouchered specimens representing both larval and metamorphic stages to assess Pr prevalence across multiple sites better.

Our results demonstrate an overall high prevalence of infections within both species across their geographic distributions, further supporting

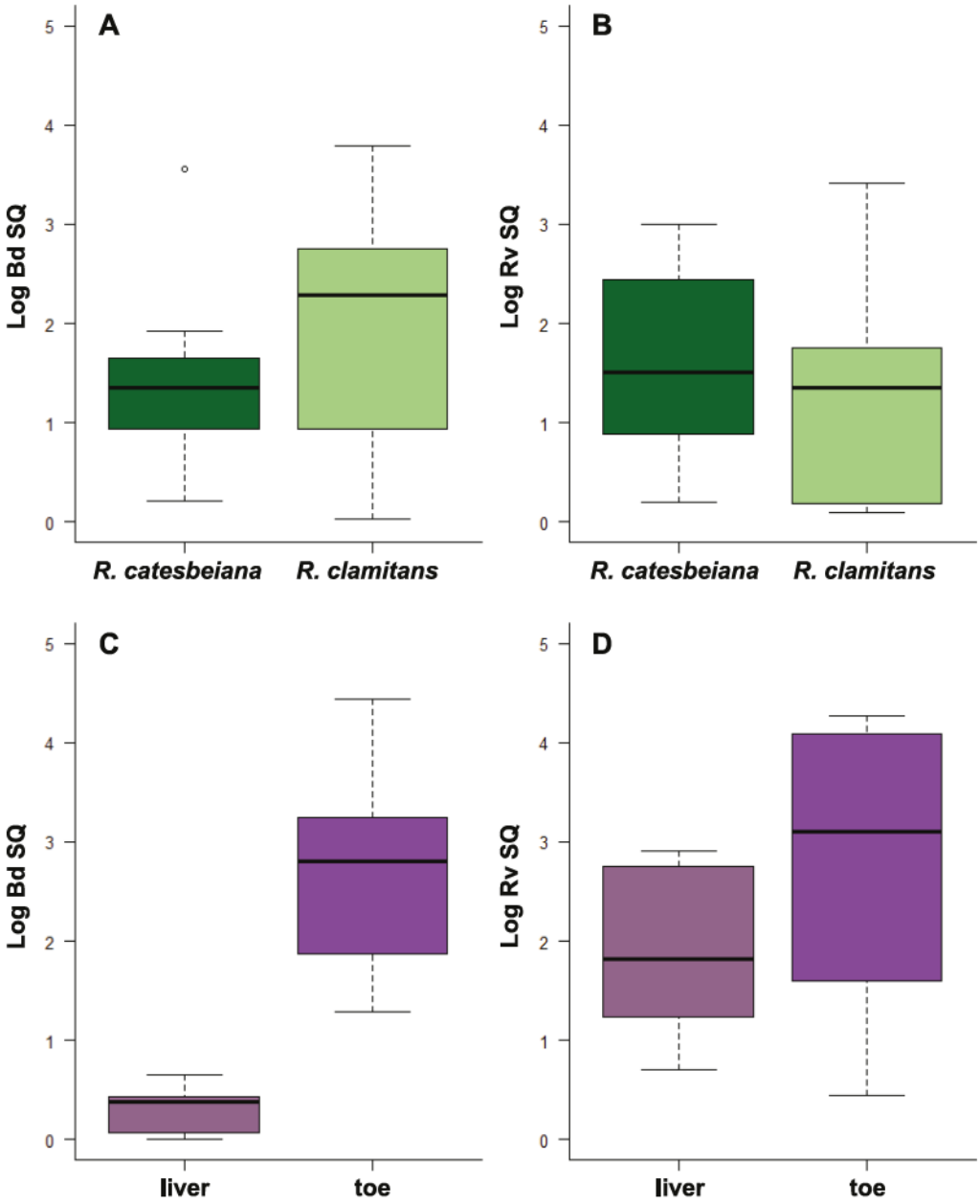

FIGURE 2. Box plots describing *Batrachochytrium dendrobatidis* (Bd) and ranavirus (Rv) infection intensity (log mean SQ). Panels A and B compare infection intensities from the American bullfrog (*Rana catesbeiana*; Bd, n=27; Rv, n=13) and green frog (*Rana clamitans*; Bd, n=29; Rv, n=8). Panels C and D compare the infection intensities from paired infected liver and toe for both species combined (Bd, n=11; Rv, n=6).

ranid species as potential pathogen reservoirs across multiple sites in the US (Yap et al. 2018). Additionally, our screening effort highlights the potential for detecting co-infections of multiple pathogens across communities. Crucial research is needed to evaluate the potential compounding negative effects of co-infections on wild anuran populations (e.g., Atkinson and Savage 2023). Access to samples of multiple tissue types and life stages is key to maximizing detection of different pathogens, enabling better understanding of their impacts on wildlife populations.

This study investigated amphibian host–pathogen detection dynamics to consider how museum tissue collections can facilitate access to resources needed for wildlife disease studies. Extended specimens that are easily accessible in natural history collections are essential for gathering data on host–pathogen interactions, along with morphology, geographic distributions, evolutionary history, and genomics. Additionally, physical and digital archives enable scientists to study the effects of disease and climate change by providing a vast record of diverse species sampled across space and time. Our findings demonstrate that having access to multiple tissue types extracted from the same individual had a notable impact on the overall number of positive infections detected. Future topics that should be investigated using museum tissue collections include pathogen detection from tissues stored using different preservation methods, such as flash-freezing in liquid nitrogen and storage in different media. Ecological research is rapidly changing, and optimizing specimen collection and preservation practices will help create detailed records of biodiversity that are most useful for current and future comparative studies.

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