



Policy analysis

One hundred years of infection with three global pathogens in frog populations of Florida, USA

Emily E. Karwacki, Katherine R. Martin, Anna E. Savage*

University of Central Florida, Department of Biology, 4110 Libra Dr, Orlando, FL 32816, United States of America



ARTICLE INFO

Keywords:

Anuran
Chytrid
Ranavirus
Perkinsea
Florida

ABSTRACT

Batrachochytrium dendrobatidis (*Bd*), *Perkinsea* (*Pr*), and *Ranavirus* (*Rv*) are three leading pathogens linked to amphibian declines, but much remains unknown about their origins and spread. Museum specimens are a critical resource for resolving epidemiological patterns because they can elucidate infection dynamics and their relationship with disease outbreaks. Here, we established a timeline of *Bd*, *Pr*, and *Rv* presence in anurans in Florida, USA, a region of high amphibian diversity. We measured pathogen prevalence and pathogen intensity in museum specimens of the genus *Rana*, a widely infected host lineage. We also sequenced barcoding regions to determine whether genetic lineages varied over time. Before this study, the first documented cases of *Bd*, *Pr*, and *Rv* in Florida were in 2009, 2002, and 2002, respectively. We detected all three pathogens nearly a century earlier, with the earliest *Bd* and *Pr* detections in 1928 and the earliest *Rv* detection in 1922. Prevalence and intensity varied across host species, geographic regions, life stages, and decades, with unique patterns for each pathogen. While we were unable to recover robust sequence data for *Rv*, the *Bd* and *Pr* sequence data showed no genetic variation over time. We found significantly more co-detection of *Pr* and *Rv* compared to any other pathogen combination, and to our knowledge, we also detected the first simultaneous tri-detection of *Bd*, *Pr*, and *Rv*. Overall, this study represents considerable extension of the timing and understanding of amphibian pathogens in the southeastern USA.

1. Introduction

Amphibians globally are experiencing mass mortality events and population declines, and infectious diseases are linked to them (Alroy, 2015; Daszak et al., 1999; Green et al., 2002; Scheele et al., 2019). Although numerous pathogens can infect amphibians, three have been associated with global anuran population decline: the fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*; Green et al., 2002; Scheele et al., 2019), the protist *Perkinsea* (hereafter *Pr*; Isidoro-Ayza et al., 2017), and the virus *Ranavirus* (hereafter *Rv*; Miller et al., 2011; While et al., 2006). Extensive research on these pathogens has revealed mechanisms of virulence, characterized global distributions, and described host-pathogen dynamics (e.g., Berger et al., 2016; Cunningham, 2018; Duffus et al., 2015; Isidoro-Ayza et al., 2017; Savage and Zamudio, 2011). However, we still lack a comprehensive understanding of where these pathogens arose and diversified, whether and when they spread to new regions, and how they became pathogenic in natural systems (Farrer et al., 2011; Isidoro-Ayza et al., 2017; Jancovich et al., 2005; Lips, 2011; O'hanlon et al., 2018). Given the continuing decimation of amphibian

populations around the world (Berger et al., 2016; Duffus et al., 2015; Scheele et al., 2019; Skerratt et al., 2007) and these remaining knowledge gaps, use of continually advancing molecular methods may be a critical approach for gaining a comprehensive understanding of amphibian diseases and preserving the inherent ecological value of amphibians (Wake and Vredenburg, 2008; While et al., 2006).

Bd is a fungal pathogen that causes the disease chytridiomycosis and primarily leads to mortality in metamorphosed anurans (Berger et al., 1998; Longcore et al., 1999). The flagellated zoospore embeds in the host's skin and causes hyperkeratinization which can negatively affect cardiac function, osmotic balance, and electrolyte balance (Berger et al., 1998; Voyles et al., 2007). *Bd* can also impair lymphocyte responses thereby evading the host immune response (Fites et al., 2013), which may be one of the reasons the pathogen has been able to spread across the globe so rapidly. *Bd* has been documented as a factor in many species extinctions and population declines globally, particularly in North and South America (Burrowes and De la Riva, 2017a; Carvalho et al., 2017; Fisher et al., 2009; Green et al., 2002; Isidoro-Ayza et al., 2017), and particularly in the Ranidae, Hylidae and Bufonidae families (La Marca

* Corresponding author.

E-mail address: anna.savage@ucf.edu (A.E. Savage).

et al., 2005; Rothermel et al., 2008; Scheele et al., 2019; Van Rooij et al., 2015; Watters et al., 2018). *Pr* is an emerging protist pathogen that primarily infects larval and juvenile frogs in the family Ranidae (Cook, 2008; Davis et al., 2007). Based on limited genetic data, *Pr* is an alveolate that is most similar to the bivalve pathogen *Perkinsus*, but it remains taxonomically undescribed (Chambouvet et al., 2015; Davis et al., 2007; Isidoro-Ayza et al., 2017; Karwacki et al., 2018; Landsberg et al., 2013). *Pr* has spore and zoospore life stages, the latter of which penetrates anuran embryos, hatchlings, and tadpoles, or is ingested through the water column by pre- and post-metamorphic anurans (Cook, 2008; Davis et al., 2007). The zoospore embeds in the host's liver, spreads throughout the internal organs, and degrades host tissue leading to organ failure (Davis et al., 2007; Green et al., 2003). *Rv* is an iridovirus that primarily affects larval amphibians and recent metamorphs, as well as fish and reptiles (Gantress et al., 2003; Greer et al., 2005). *Rv* infects the host's internal organs and causes internal and external hemorrhaging, edemas, and multi-organ necrosis (Greer et al., 2005; Miller et al., 2008). *Rv* has caused numerous mass mortality events, mostly in North America and Europe, usually with sudden onsets and high casualties (Duffus et al., 2015; Green et al., 2002; Koo et al., 2017; Miaud et al., 2016; Rijks et al., 2016; Smith et al., 2016). Similar to *Bd* and *Pr*, *Rv* commonly infects species in the Ranidae and Hylidae families (Duffus et al., 2015; Johnson and Hoverman, 2012; Smith et al., 2016; Watters et al., 2018). Research on all three of these pathogens has led to a growing concern about the loss of anuran populations due to infectious diseases, and a need for more thorough epidemiological understanding.

Despite extensive global research on *Bd*, *Pr*, and *Rv*, few studies have examined their infection dynamics in the state of Florida, USA, despite this state harboring the second largest number of frog species in the country (only behind Texas; Moriarty, 2017), including 8 native ranid frogs and 14 native hylids (Ashton and Ashton, 1988). The earliest documented cases of presence or infection by *Bd*, *Pr*, and *Rv* in any anuran in Florida are 2009 (Rizkalla, 2009), 2002 (Rothermel et al., 2008), and 2002 (Isidoro-Ayza et al., 2017) respectively. *Bd* has been found in amphibian populations throughout the southeastern U.S. (Green and Dodd, 2007; Ouellet et al., 2005; Rothermel et al., 2008) though with little documented presence in Florida (Horner et al., 2017; Rizkalla, 2009). *Pr* has been documented in southeastern U.S. anuran populations (Isidoro-Ayza et al., 2017) and recent studies have shown *Pr* present in northern and central Florida with high prevalence and infection intensity in some ranid and hylid species (Isidoro-Ayza et al., 2017; Karwacki et al., 2018; Landsberg et al., 2013), though limited sampling has been conducted in southern Florida. *Rv* also infects ranid and hylid species in the southeastern U.S. (Duffus et al., 2015; Gray et al., 2009; Horner et al., 2017). To date, *Rv* has only been detected at single sites in northern (Landsberg et al., 2013), central (Horner et al., 2017), and southern Florida (Isidoro-Ayza et al., 2017). Considering the lethality of these pathogens and the presence of many putatively susceptible host populations in Florida, this region merits further research on amphibian disease presence and infection dynamics.

One way in which studies of pathogen origin, evolution, and spread can be improved is by using historical museum specimens preserved in formalin and housed in natural history museums and collections. By leveraging new molecular techniques and applying them to preserved specimens we can garner valuable information about species and populations as well as genetic, host, and regional shifts throughout time in relation to disease (Adams et al., 2017; Ouellet et al., 2005; Puschendorf et al., 2006; Talley et al., 2015). Specifically, we can use existing data such as climate records, pet trade routes, and industrialization dates in combination with time series of specimens and their pathogens to make new observations and discoveries on pathogen mechanics (Burrowes & De La Riva 2017a; Burrowes and De la Riva, 2017b; Cheng et al., 2011; Lips, 2011; Puschendorf et al., 2006). For example, Talley et al. (2015) used museum specimens of anurans from three different natural history collections to show that *Bd* had been present in ranid frog populations in Illinois since at least 1888. Similarly, Burrowes and De la

Riva (2017a) used museum specimens to show that *Bd* was present in Bolivian anuran populations prior to the occurrence of any 21st century mass mortality event. These studies highlight how collections-based epidemiological studies can reveal pathogen presence in a population much earlier than when disease outbreaks have been documented (La Marca et al., 2005). By leveraging museum collections with data on global distribution, historical climate, and mass mortality events, clearer and broader epidemiological patterns can be formed and conservation efforts can move forward (Burrowes and De la Riva, 2017a; Carvalho et al., 2017; Cheng et al., 2011; Puschendorf et al., 2006; Zhu et al., 2014). However, this can only happen when museum collections are regularly curated, well managed, and well-funded (Burrowes and De la Riva, 2017b; Heberling et al., 2019). The fires at the National Museum of Natural History in New Delhi (India) in April of 2016 and the Museu Nacional (Brazil) in September of 2018, and the subsequent loss of biological and anthropological data, highlight the importance of adequate management and funding in order to maintain and protect natural history collections.

Ancient DNA sequencing of museum collections provides valuable information about pathogen origins and evolutionary history. Sequence data can show whether pathogen genomes have evolved over time and can confirm minimum earliest dates of pathogen presence in certain regions or host species. However, generating sequence data from specimens is difficult if they are exceptionally old or have been formalin-fixed, a standard procedure for amphibians until recently. Because formaldehyde degrades DNA over time, typically only short (<200 base pair) sequence fragments can be recovered (Schander and Halanych, 2003) or sequences may not be recoverable at all (Huss et al., 2014). Due to the challenges inherent to sequencing short fragments of ancient DNA and the high risk of contamination by modern DNA, the optimal approach to obtain DNA from formalin-fixed specimens is to extract DNA in a sterile environment that is physically isolated from any modern DNA, to use multiple controls in each stage of molecular work, and to repeat experiments independently to minimize mistaking DNA contamination for novel sequence data (Cooper and Poinar, 2000; Wandeler et al., 2007). Despite these challenges, ancient DNA techniques have advanced considerably (Burrowes and De la Riva, 2017b; Seimon et al., 2015; Weldon et al., 2004) and should continue being utilized for epidemiological studies.

Here we used museum collections and ancient DNA techniques to establish geographic and temporal patterns of *Bd*, *Pr*, and *Rv* detections in Florida anurans from 1922 to the present, including where, when, and in which species and life stages disease detections were most prevalent and intense. We focused on specimens in the genus *Rana* (family Ranidae) from the Florida Museum of Natural History (FMNH) collections because this genus is widespread throughout Florida, and is also among the most susceptible to all three pathogens (Duffus et al., 2015; Green et al., 2002; Isidoro-Ayza et al., 2017; Landsberg et al., 2013). Additionally, two of the seven species studied are listed as vulnerable or declining, while the other species are commonly threatened by further urban development throughout the state ("The IUCN Red List of Threatened Species," 2020). Our study provides the first broad-scale characterization of major amphibian pathogens present in Florida anurans over the past century.

2. Methods

2.1. Sample collection

Anuran specimens were sampled from the herpetology collection at the Florida Museum of Natural History (Gainesville, Florida, USA) because this museum harbors the world's largest collection of native Florida amphibians (<https://www.floridamuseum.ufl.edu/herpetology/>). We sampled specimens within the frog genus *Rana*, each of which had been formalin-fixed and stored in ethanol at the museum. All specimens were collected in Florida, USA, from 60 of the 67 currently delineated

counties (Fig. 1A-C) and no specimens had outward signs of disease at the time of sampling. To broadly assess latitudinal patterns, given that significant climate and ecological differences occur within Florida (Fovell and Fovell, 1993), we combined counties into three regions: north, central, and south Florida. Collection jars were organized by species and county. Some jars contained single specimens, but more than half contained two or more specimens. In most cases, all frogs from a jar were swabbed however, when there were 50 or more in a jar, which could introduce bias towards a certain county, decade, or species, only half of those specimens were swabbed. Out of 130 total specimen jars, two contained individuals from two different counties, three contained two species, and the remaining 125 contained a single species from a single county. Specimen collection dates ranged from 1922 to 2017. We sampled pre- and post-metamorphic anurans because different life stages can harbor distinct infection patterns (Carvalho et al., 2017). Specimens were swabbed (Huss et al., 2014; Seimon et al., 2015) and tested via quantitative (q)PCR for all three pathogens (Burrowes and De la Riva, 2017a; Cheng et al., 2011), and those that had high pathogen intensities were run independently via traditional PCR and the product was Sanger sequenced. Recovered sequences were compared to modern pathogen sequences to assess pathogen lineages in Florida, as well as to confirm pathogen identity. In order to control for possible frog-to-frog pathogen contamination during storage in the jars, swabs of each jar were also taken by thoroughly running a sterile medical swab (Medical Wire & Equipment Co., MW113) along the upper and middle part of the inside wall three times. Though having multiple specimens in a jar does not always mean contamination will happen (Richards-Hrdlicka, 2012), we used these jar controls to be more conservative in our interpretation of the results. Individual frog specimens were also swabbed using sterile medical swabs following established amphibian pathogen practices (Hyatt et al., 2007). Specifically, each post-metamorphic individual was swabbed using 25 strokes (5 strokes on each rear foot, the abdomen, and the inner thighs), and each pre-metamorphic individual was swabbed using 20 strokes (5 strokes on each side of the tail, the mouth parts, and

the abdomen; Soto-Azat et al., 2009). All swabs were immediately placed in individual sterile dry 2 mL tubes and stored in a -20°C freezer until extraction. Gloves were worn while swabbing and were changed between each frog to prevent contamination with modern DNA, and contamination between jars.

2.2. Quantitative (q)PCR

DNA was extracted from each swab using DNeasy Blood and Tissue Kits (Qiagen) according to the manufacturer's protocol, which is recommended for ethanol-preserved and formalin-fixed specimens (Adams et al., 2015). We made one modification to improve extraction of DNA from swabs: each swab was faced cotton-side up when moved into spin columns to ensure the cotton didn't block chemical flow-through. Each extract was stored in a -20°C freezer until analysis. Each sample was analyzed using pathogen-specific qPCR assays in order to determine presence/absence and to quantify the pathogen intensity for *Bd* (Boyle et al., 2004), *Rv* (Allender et al., 2013), and *Pr* (Karwacki et al., 2018). All samples were run with a total reaction volume of 25 μL as follows: 8 μL of $1 \times$ concentration Supermix (Bio-Rad), 2 μL of each primer ($10 \mu\text{M}$; Table S1), 5 μL of probe ($1 \mu\text{M}$; Table S1), 3 μL of molecular-grade water, and 5 μL of template DNA. All qPCRs were run on a CFX96 Real-Time System (Bio-Rad), and analyzed using Bio-Rad CFX Manager software. Serial dilutions of gBlocks (Integrated DNA Technologies; Table S1) specific for each pathogen's target DNA region were run in duplicate on every qPCR plate. Serial dilutions for *Pr* ranged from 2×10^8 to 2×10^0 gene copies/ μL , dilutions for *Bd* ranged from 2×10^6 to 2×10^{-1} gene copies/ μL , and dilutions for *Rv* ranged from 1×10^9 to 1×10^0 gene copies/ μL . Each plate included at least two negative controls (consisting of molecular grade water) to ensure no contamination was present in the reagents. All reactions were run with the following protocol: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, and 60°C (for *Bd* and *Rv*) or 59°C (for *Pr*) for 1 min. Each sample was run twice, and any samples that amplified at significantly different (more than an order of

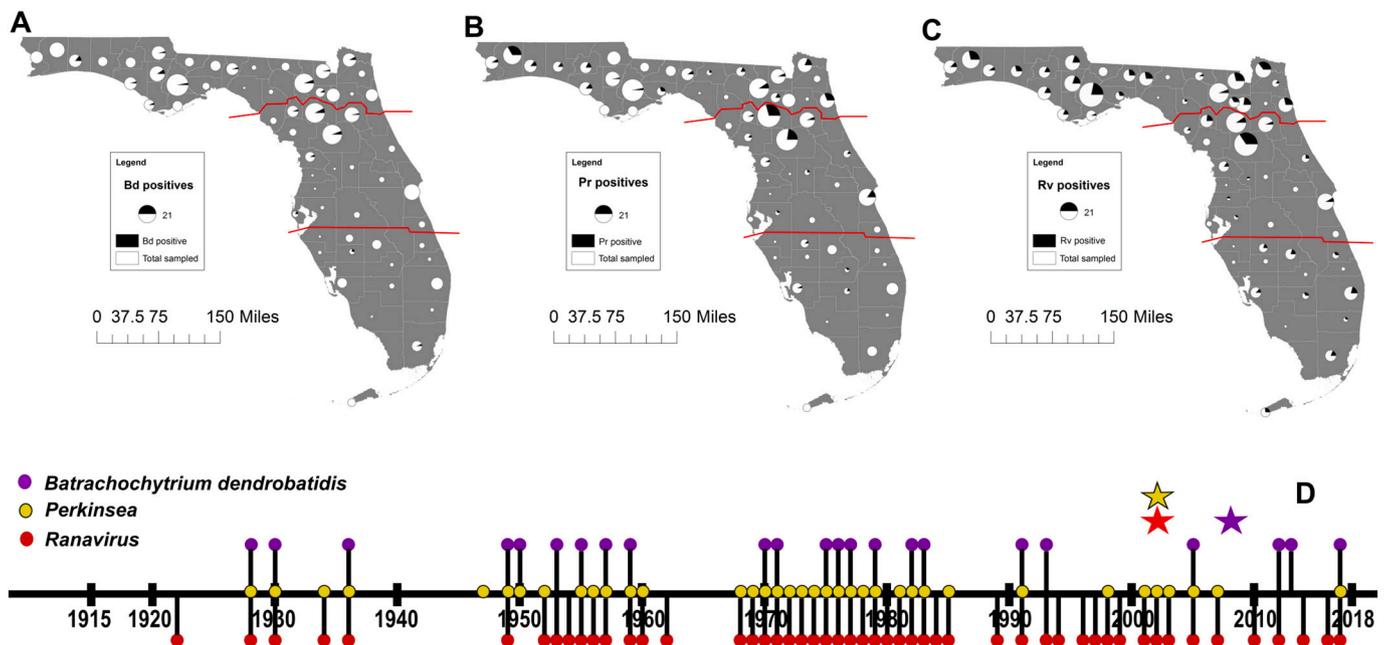


Fig. 1. Map of Florida, USA showing A) *Bd* disease prevalence, B) *Pr* disease prevalence, and C) *Rv* disease prevalence. The state is separated into northern, central, and southern regions by the red lines. Pie charts represent pathogen prevalence; white coloring represents negative samples while black coloring represents positive samples, and pie charts are proportional to sample size from that county with the chart in the legend referencing population size. For each pie chart a central latitude and longitude point were used for each county sampled. D) A one-hundred-year timeline representing positive cases. *Bd*-positive samples are indicated in purple at the top, *Pr*-positive samples are indicated in yellow in the middle, and *Rv*-positive samples are in red at the bottom of the line. Stars represent the first documented cases of each pathogen's presence in Florida prior to this study (2002 for *Pr* and *Rv*, and 2009 for *Bd*), and black rectangles represent years when tri-detection occurred (1928, 1975, and 1991). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

magnitude) cycles were run a third time. The two most similar values were then averaged and multiplied by 40 to estimate the total pathogen intensity in the entire 200 μ L swab extraction. Conservatively, samples were only considered true positives if they amplified before cycle 36 for all three qPCR protocols.

If control jar swabs amplified during qPCR, then a specimen that amplified from the same jar could not always confidently be considered positive (instead, it could represent contamination from another specimen in that jar). Thus, when a control sample amplified, only specimens which amplified with a pathogen intensity at least an order of magnitude higher than the jar swab were considered true positives. In these cases, only the positive specimen collected most recently was used as the year of the positive result to be conservative about dating how long ago these pathogens were detected in each species and region.

2.3. Validation

To confirm pathogen identity, samples with >4000 gene copies from qPCR analyses were PCR-amplified utilizing the same pathogen-specific qPCR primers (Table S1). PCRs were run in 20 μ L reactions as follows: 5.6 μ L 1 \times concentration OneTaq Standard Buffer with MgCl₂ (New England BioLabs), 1.6 μ L of dNTP (10 mM), 0.4 μ L DMSO (10 mM), 0.4 μ L of each primer (10 μ M), 0.25 μ L of OneTaq Polymerase (Applied Biosystems), 2 μ L of template DNA, and 9.35 μ L of molecular grade water. *Bd* reactions were run using the following protocol: 95 °C for 5:00, followed by 40 cycles of 95 °C for 0:15, 60 °C for 1:00, and 72 °C for 1:00, with a final extension of 72 °C for 5:00. *Pr* reactions were run using the following protocol: 95 °C for 1:30, followed by 40 cycles of 95 °C for 0:30, 52 °C for 0:30, and 72 °C for 1:30, with a final extension of 72 °C for 5:00. *Rv* reactions were run using the following protocol: 95 °C for 2:00, followed by 40 cycles of 95 °C for 0:45, 56 °C for 0:45, and 72 °C for 0:45, with a final extension of 72 °C for 5:00. *Bd* primers amplified a 120 bp fragment of the rDNA internal transcribed spacer (ITS-1) region (Boyle et al., 2004), *Pr* primers amplified a 126 bp segment of the central region of the 18S ribosomal RNA gene (Karwacki et al., 2018), and *Rv* primers amplified a 54 bp region of the major capsid protein (MCP) of anuran-specific *Rv* (Allender et al., 2013). All PCR products were visualized on a 2% agarose gel, and product of the correct fragment size was sent for Sanger sequencing in both directions at Eurofins Genomics Facility (Louisville, Kentucky, USA). After assembling and cleaning forward and reverse sequence reads by eye, we used BLAST (Basic Local Alignment Search Tool) implemented in Geneious (BioMatters) to compare our resulting sequence data against existing sequences in GenBank.

In addition to validation by sequencing, a subset of the initial samples ($N = 48$ specimens from 21 different jars) was re-swabbed at the FMNH, along with control swabs of the jar, and again stored dry in sterile 2.0 mL tubes in a -20 °C freezer until analysis. These were taken to further validate pathogen presence. To avoid possible contamination from modern pathogen samples, the DNA from these samples was extracted in a clean, DNA-free environment. Specifically, we used a newly established pre-PCR room that had never been used for molecular work before, that was separated from other laboratory areas by a windowless locked door. Furthermore, all DNA extractions and qPCR plate preparations were performed in a brand new AC600 PCR Workstation with HEPA filter and UV irradiation (AirClean Systems) within the pre-PCR room. We UV irradiated the entire workstation before each procedure and sealed qPCR plates with optical lids before they were removed from the workstation. Finally, the plates were run on a qPCR machine in a core facility separate from our main laboratory space as a final precaution against contamination.

2.4. Statistical analyses

Using chi-square tests of independence, we tested for significant differences in the prevalence of each pathogen based on the following

variables: species, decade (1920s – 2010s), region (north, central, or south Florida), and life stage (pre-metamorphic or post-metamorphic, defined as tadpoles without limbs and frogs of any size with fully developed limbs, respectively). We also tested for significant differences in the proportions of pairwise pathogen co-detections (*Bd-Rv*, *Pr-Rv*, and *Bd-Pr* co-detections), the proportion of total individuals positive for each pathogen, and the proportion of re-swabs with consistent detection status across species and pathogens. Chi-square tests were implemented in RStudio version 3.6.1 using the `chisq.test` function in the base R stats package in (R Core Team, 2019). For any grouping for which the calculated expected value was less than one, that group was removed from the global chi-square test and subsequent post-hoc analyses (Campbell, 2007). After global chi-square tests, significant associations were explored with pairwise comparisons between pairs of proportions using the `pairwise.prop.test` function in the base R stats package in RStudio version 3.6.1 (R Core Team, 2019) using false discovery rate *P*-value correction to account for inflated Type I error rate due to multiple comparisons.

We separately modeled prevalence and intensity of each pathogen as a function of species, decade (1920s – 2010s), life stage (pre-metamorphic or post-metamorphic), and region (north, central, or south Florida). Pathogen prevalence and intensity was modeled using fixed effect generalized linear models and fixed effect linear models, respectively. To explore these relationships, we modeled prevalence and intensity first as a function of each variable independently, and then as a function of all additive combinations of variables. Finally, given that the sampled frog species are not evenly distributed throughout regions of Florida, we also explored models with an interaction term between species and region. We implemented these analyses in RStudio version 3.6.1 with the `lm` and `glm` functions in the base R stats package (R Core Team, 2019). We used multiple combinations of covariates to model pathogen prevalence and intensity, and compared models using AIC_c with the R package `bbmle` using the `AICcTab` function (Bolker and Core Team, 2017) to determine the most plausible models. To arrive at the most plausible models explaining pathogen dynamics, we created a confidence set of models by starting at the best performing model (i.e. the highest AIC_c weight) and proceeded down the list until the cumulative Akaike weight exceeded 0.95 (Symonds and Moussalli, 2011).

3. Results

We swabbed seven ranid frog species ($N = 806$, Table 1), including *Rana catesbeiana* (American bullfrog, $N = 62$), *Rana clamitans* (green frog, $N = 54$), *Rana grylio* (pig frog, $N = 97$), *Rana heckscheri* (river frog, $N = 201$), *Rana okaloosae* (Florida bog frog, $N = 18$), *Rana sphenoccephala* (southern leopard frog, $N = 345$), *Rana virgatipes* (carpenter frog, $N = 26$), and three unidentified specimens where species was not recorded at the time of capture ($N = 3$). Of the 131 jars swabbed as controls, two were positive for *Bd*, 29 were positive for *Pr*, and 23 were positive for *Rv*.

Only specimens for which species, decade, region, and life stage information was available were retained in the following statistical analyses ($N = 780$). At least one of the three focal pathogens was detected in 282/780 (36.15%) of sampled specimens, including 31 *Bd* positive specimens, 113 *Pr* positive specimens, and 194 *Rv*-positive specimens. Among specimens with single pathogen detections, seven had *Bd* only, 60 had *Pr* only, and 136 had *Rv* only. Among specimens with two pathogens, eight had *Bd-Pr* co-detections, 13 had *Bd-Rv* co-detections, and 42 had *Pr-Rv* co-detections. Finally, three specimens had tri-detection of all three pathogens. Pathogen prevalence differed significantly among the three pathogens ($\chi^2_{(df=2, N=780)} = 138, p < 0.001$ with higher *Rv* prevalence (0.25) compared to *Pr* (0.14; $p < 0.001$) or *Bd* (0.04; $p \leq 0.001$), and higher *Pr* prevalence compared to *Bd* ($p < 0.001$).

Bd was detected in 3.97% of swabbed specimens. *Bd* was detected in multiple individuals from every region within Florida, including at least two specimens from each decade (Table 1, Fig. 1A, 1D). The oldest specimen positive for *Bd* was a *R. heckscheri* individual from north

Table 1

Number of *Ranidae* samples swabbed across decade and species tested. Positive pathogen proportions are listed below the total of each species. Additional samples with no documented collection year total 24 individuals for a total $N = 806$.

Species	Disease	Decade										Total
		1920	1930	1940	1950	1960	1970	1980	1990	2000	2010	
<i>R. catesbeiana</i>	Total	0	2	2	17	12	8	7	7	2	1	58
	<i>Bd</i>	0/0	0/2	0/2	0/17	0/12	0/8	0/7	1/7	0/2	0/1	
	<i>Pr</i>	0/0	1/2	0/2	4/17	4/12	1/8	4/7	1/7	0/2	0/1	
	<i>Rv</i>	0/0	1/2	0/2	8/17	1/12	5/8	4/7	1/7	1/2	0/1	
<i>R. clamitans</i>	Total	0	0	1	3	7	21	14	4	2	1	53
	<i>Bd</i>	0/0	0/0	0/1	0/3	0/7	2/21	0/14	0/4	0/2	0/1	
	<i>Pr</i>	0/0	0/0	1/1	0/3	1/7	6/21	4/14	1/4	0/2	0/1	
	<i>Rv</i>	0/0	0/0	0/1	0/3	1/7	3/21	3/14	1/4	1/2	0/1	
<i>R. gryllo</i>	Total	1	8	1	39	0	27	2	7	2	5	92
	<i>Bd</i>	0/1	1/8	0/1	2/39	0/0	3/27	0/2	1/7	0/2	0/5	
	<i>Pr</i>	0/1	3/8	1/1	4/39	0/0	2/27	0/2	1/7	0/2	0/5	
	<i>Rv</i>	1/1	1/8	1/1	15/39	0/0	5/27	0/2	3/7	1/2	2/5	
<i>R. heckscheri</i>	Total	8	1	10	84	1	48	25	10	7	3	197
	<i>Bd</i>	2/8	0/1	1/10	2/84	0/1	2/48	1/25	1/10	2/7	1/3	
	<i>Pr</i>	4/8	0/1	1/10	4/84	0/1	15/48	9/25	0/10	4/7	0/3	
	<i>Rv</i>	7/8	0/1	1/10	13/84	0/1	11/48	10/25	6/10	2/7	2/3	
<i>R. okaloosae</i>	Total	0	0	0	0	0	0	18	0	0	0	18
	<i>Bd</i>	0/0	0/0	0/0	0/0	0/0	0/0	3/18	0/0	0/0	0/0	
	<i>Pr</i>	0/0	0/0	0/0	0/0	0/0	0/0	10/18	0/0	0/0	0/0	
	<i>Rv</i>	0/0	0/0	0/0	0/0	0/0	0/0	5/18	0/0	0/0	0/0	
<i>R. sphenoccephala</i>	Total	1	32	16	45	38	92	25	26	26	37	338
	<i>Bd</i>	0/1	1/32	1/16	2/45	0/38	0/92	0/25	0/26	0/26	2/37	
	<i>Pr</i>	0/1	13/32	0/16	0/45	0/38	8/92	0/25	2/26	2/26	1/37	
	<i>Rv</i>	0/1	6/32	3/16	11/45	11/38	20/92	4/25	6/26	3/26	5/37	
<i>R. virgatipes</i>	Total	0	0	0	0	2	20	4	0	0	0	26
	<i>Bd</i>	0/0	0/0	0/0	0/0	0/2	0/20	0/4	0/0	0/0	0/0	
	<i>Pr</i>	0/0	0/0	0/0	0/0	0/2	1/20	0/4	0/0	0/0	0/0	
	<i>Rv</i>	0/0	0/0	0/0	0/0	0/2	9/20	0/4	0/0	0/0	0/0	
Total		10	43	30	188	60	216	95	54	39	47	782

Florida collected in 1928 (Fig. 1D). *Bd* was detected in six of the seven species tested, and in every decade except the 1960s. For chi-square tests, *R. okaloosae* and *R. virgatipes* were removed and specimens collected in the 1920s were removed due to insufficient expected value size. *Bd* prevalence differed significantly across the remaining species ($\chi^2_{(df=4, N=736)} = 11, p = 0.026$), although no species pairs were significantly different from one another in pairwise comparisons (Table S2). *Bd* prevalence was not significantly different across decades ($\chi^2_{(df=8, N=770)} = 5.1, p = 0.75$), regions ($\chi^2_{(df=2, N=780)} = 0.97, p = 0.62$), or life stages ($\chi^2_{(1, N=780)} < 0.001, p = 0.99$). The confidence set of five models that best explained *Bd* prevalence included species, region, life stage, and in one model (weight = 0.018) decade (Table S7). The confidence set of four models that best explained *Bd* intensity included region, life stage, and species (Table S8).

Pr was detected in 14.49% of swabbed specimens. Multiple individuals were found positive for *Pr* from every region within Florida, and at least three specimens from each decade and species were positive (Table 1, Fig. 1B, 1D). The oldest specimen positive for *Pr* was a *R. heckscheri* from north Florida collected in 1928 (Fig. 1D). *Pr* prevalence was significantly different across species ($\chi^2_{(df=6, N=780)} = 53, p < 0.001$; Table S2), decade ($\chi^2_{(df=9, N=780)} = 61, p < 0.001$), and region ($\chi^2_{(df=2, N=780)} = 19, p < 0.001$). Furthermore, the proportion of pre-metamorphic individuals detected with *Pr* (0.20) was significantly higher than that of post-metamorphic individuals (0.13; $\chi^2_{(df=1, N=780)} = 4.8, p = 0.029$). Several species pairs differed significantly, with *Pr* prevalence significantly lower in *R. sphenoccephala* compared to most other species (Fig. 2, Table S3). *Pr* prevalence was significantly higher in the 1930s compared to most other decades, and significant differences were found in several decade comparisons (Fig. 2, Table S4). Prevalence was significantly different in all three Florida regions, with *Pr* prevalence lower in south (0.03) versus central (0.22; $p \leq 0.001$) and north (0.13; $p = 0.011$) Florida, and lower in north versus central ($p = 0.009$) Florida. The confidence set of five models that best explained *Pr* prevalence included species, region, life stage, decade, and the interaction

between species and region (Table S9). In contrast, *Pr* intensity was best explained by a single model (AIC weight = 0.98) that included species, region, life stage, and the interaction between species and region (Table S10).

Rv was detected in 24.87% of swabbed specimens. Multiple individuals were found positive for *Rv* from every region within Florida, and at least five specimens from each decade were positive (Table 1, Fig. 1C, 1D). The oldest specimen positive for *Rv* was a *R. gryllo* from central Florida collected in 1922 (Fig. 1D). *Rv* prevalence was not significantly different across region ($\chi^2_{(df=2, N=780)} = 2.5, p = 0.290$) or life stage ($\chi^2_{(df=1, N=780)} = 0.22, p = 0.640$). In contrast, *Rv* prevalence differed significantly across species ($\chi^2_{(df=6, N=780)} = 13, p = 0.040$) but no pairwise species comparisons were significant after correcting for multiple comparisons (Table S5). *Rv* prevalence also differed significantly across decade ($\chi^2_{(df=9, N=780)} = 21, p = 0.011$). Specifically, *Rv* prevalence was significantly higher in the 1920s (0.80) than in every other decade except the 1990s (Table S6). No variables clearly explained *Rv* prevalence, as the confidence set included 15 models with different combinations of all variables (Table S11). The confidence set of six models that best explained *Rv* intensity included different combinations of species, region, life stage, the interaction between species and region, and in one model (weight = 0.049), decade (Table S12).

We recovered 63 individuals that were positive for two pathogens, including eight individuals that were positive for *Pr* and *Bd*, 42 individuals that were positive for *Pr* and *Rv*, and 13 individuals that were positive for *Rv* and *Bd*. Most of these individuals were *R. heckscheri* ($N = 25$) or *R. sphenoccephala* ($N = 10$). The proportion of co-detections differed significantly among pathogen combinations ($\chi^2_{(df=2, N=63)} = 32, p < 0.001$). Specifically, post-hoc pairwise comparisons found that *Pr-Rv* co-detections (0.67) occurred significantly more often than either *Pr-Bd* co-detections (0.13; $p < 0.001$) or *Rv-Bd* co-detections (0.21; $p < 0.001$), whereas the proportion of *Pr-Bd* and *Rv-Bd* co-detections were not significantly different from each other ($p = 0.280$). We also detected the presence of three pathogens simultaneously in three individual

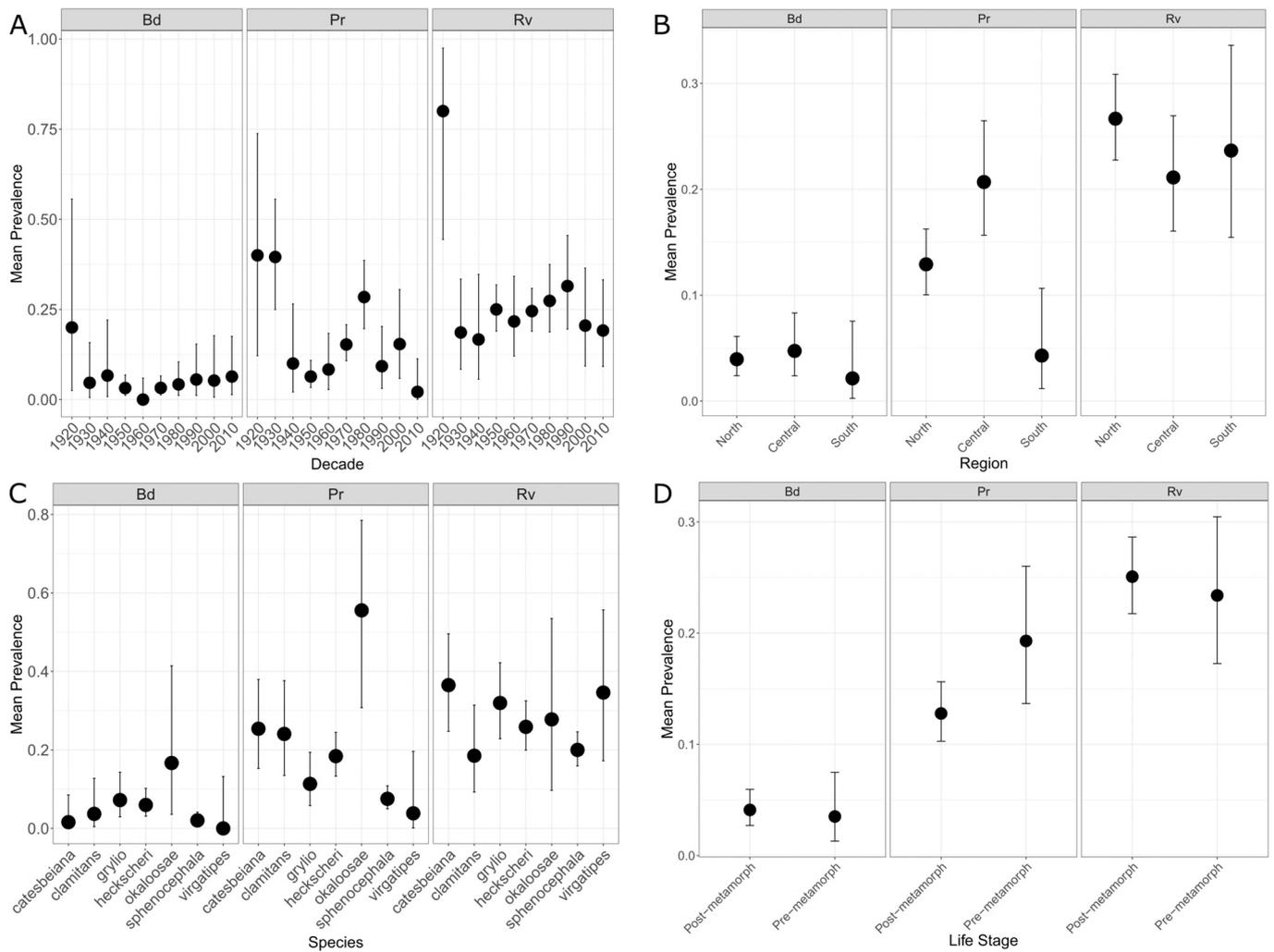


Fig. 2. Mean prevalence of *Bd*, *Pr*, and *Rv* ($\pm 95\%$ Clopper-Pearson confidence intervals) shown across A) decades, B) regions of Florida, C) species, and D) life stages.

specimens: one *R. heckschleri* collected in 1928 from north Florida, one *R. heckschleri* collected in 1975 from central Florida, and one *R. catesbeiana* collected in 1991 from central Florida (Fig. 1D).

Clean sequence data was recovered from four *Bd*-positive specimens out of six sent for sequencing, and from three *Pr*-positive specimens out of 13 sent for sequencing, further suggesting that swabs are an effective method of pathogen detection. Of the 12 *Rv*-positive specimens sent for sequencing, all 12 were unsuccessful. Sequences recovered for *Bd* were from frogs collected in 1971, 1973, 1983, and 1991 (Fig. S1). Sequences recovered for *Pr* were from frogs collected in 1928, 1936, and 1956 (Fig. S2). BLAST searches demonstrated that our *Bd* sequences were 93–100% identical to other *Bd* sequences in GenBank, specifically those in the Global Pandemic Lineage (*Bd*-GPL; Mutnale et al., 2018; Schloegel et al., 2012; Schoch et al., 2014; Wang et al., 2018; Fig. S1) and *Pr* sequences were 95–100% identical to other *Pr* sequences in GenBank (Isidoro-Ayza et al., 2017; Karwacki et al., 2018; Fig. S2).

The subset of specimens that were re-swabbed and run in the clean room matched the initial qPCR results at different rates for each pathogen. Of this set of specimens, ten were *R. catesbeiana*, 18 were *R. clamitans*, 19 were *R. grylio*, and one was *R. heckschleri*, and all but one of these specimens were post-metamorphic (Table 2). For *Bd*, samples matched the original amplifications 94.20% (65/69) of the time, and there were no significant differences across species in re-swab consistency ($\chi^2_{(df=1, N=37)} < 0.001, p = 1$). For the four samples that inconsistently amplified for *Bd*, all were negative for the initial swab and positive for the reswab. For *Pr*, samples matched 57.97% (40/69) of the

Table 2

Proportion of positive samples that were also positive when re-swabbed and re-run, separated by species and pathogen.

Species	Reswab matches		
	<i>Bd</i>	<i>Pr</i>	<i>Rv</i>
<i>R. catesbeiana</i>	10/10	9/10	6/10
<i>R. clamitans</i>	17/18	13/18	12/18
<i>R. grylio</i>	17/19	6/19	6/19
<i>R. heckschleri</i>	1/1	0/1	0/1
Total	45/48	28/48	24/48

time, and there were significant differences across species in re-swab consistency ($\chi^2_{(df=2, N=47)} = 11.22, p = 0.004$), with *R. catesbeiana* (0.90; $p = 0.028$) and *R. clamitans* (0.72; $p = 0.048$) showing significantly higher reswab consistency compared to *R. grylio* (0.32; *R. heckschleri* was excluded due to expected size < 1). The 29 samples that inconsistently amplified for *Pr* included eight that were positive initially and 21 that were negative initially. Finally, for *Rv*, samples matched 50.72% (35/69) of the time, and there were no significant differences across species in reswab consistency ($\chi^2_{(df=2, N=47)} = 4.96, p = 0.080$). For the 34 samples that did not match for *Rv*, 26 were positive initially and eight were negative initially.

4. Discussion

This study represents the first state-wide geographic and historic analysis of amphibian pathogens in Florida frog populations. By using ranid specimens housed at the FMNH collected from 1922 to 2017, we were able to determine the minimum earliest pathogen detections of *Bd*, *Pr* and *Rv*. We found that *Bd* and *Pr* have been present in Florida ranid populations since at least 1928, and *Rv* has been present since at least 1922 (Fig. 1D), extending the previous earliest records by almost one hundred years for all three pathogens (Isidoro-Ayza et al., 2017; Rizkalla, 2009; Rothermel et al., 2008). We also found more frequent instances of co-detection than previously noted among these pathogens (Landsberg et al., 2013; Love et al., 2016; Stutz et al., 2018; Watters et al., 2018; Whitfield et al., 2013). Our study demonstrates that all three pathogens have been present in Florida for decades longer than previously documented, raising questions about the timing of original pathogen introductions and drivers of pathogenicity.

Across all pathogens, we found a trend towards higher prevalence during the 1920s and/or 1930s (Fig. 2), although prevalence was only significantly higher for *Rv* in the 1920s and *Pr* in the 1930s. We speculate these prevalence spikes could be due to the land boom that occurred until 1928, when large plots of land were developed throughout Florida, and/or the spread of pathogens from the non-native Cuban tree frog (*Osteopilus septentrionalis*) which first appeared in Florida in the 1920s (Meshaka, 2001). However, further epidemiological studies of specimens collected from other regions in the early 1900s are needed to assess whether this pattern is unique to Florida or may arise from other global trends. Environmental and anthropogenic factors have been implicated in numerous amphibian population declines linked to disease (Farrer et al., 2011; Jancovich et al., 2005; Kiesecker et al., 2001; North et al., 2015; Schloegel et al., 2009). We also know that anthropogenic factors can have drastic effects on population disease dynamics (Jancovich et al., 2005; North et al., 2015; Youker-Smith et al., 2018), though understanding to what extent historical events have influenced amphibian disease epidemiology requires further analyses. The lack of significant change in *Bd* prevalence or intensity over time detected from our dataset is particularly noteworthy, as this suggests that the strain(s) in Florida are likely endemic and that the global wave of epizootic *Bd* in the latter 20th century (Wake and Vredenburg, 2008) did not cause disease spikes in Florida. Furthermore, the persistence of ranid populations in Florida despite ongoing presence of three major pathogens for nearly a century highlights the distinction between infections and disease, and the potential that these pathogens only cause sub-lethal effects in at least some the species examined. However, we caution that Florida ranids having survived pathogen threats over the last century does not guard against negative impacts of subsequent pathogen increases or novel introductions.

Despite extensive research into the global distributions of amphibian pathogens, few studies have focused on Florida or other parts of the southeastern US, an area of extremely high amphibian biodiversity (Ashton and Ashton, 1988). Analysis of museum specimens throughout Canada and the U.S. from 1895 to 2001 showed no *Bd* presence in Florida (Ouellet et al., 2005), but the pathogen has been detected in more recent sampling events (Horner et al., 2017; Rizkalla, 2009). Although *Bd* prevalence in this study was low (< 0.21) across all comparisons (Fig. 2), our consistent detection of this pathogen over time, space and species highlights the importance of large sample sizes for documenting pathogen dynamics. While recent *Pr* and *Rv* studies in Florida have detected high pathogen prevalence, they have mostly been restricted to north and central Florida, with limited sampling conducted in the southern part of the state (Isidoro-Ayza et al., 2017; Karwacki et al., 2018; Landsberg et al., 2013). Here, we found that *Pr* and *Rv* occur throughout the state with high average pathogen intensities (Figs. 1B, 1C, and 3), which likely indicates actual infections with these pathogens and suggests the presence of diseased individuals. *Rv* prevalence was significantly higher than either *Bd* or *Pr*, consistent with other studies in

other parts of the US (e.g., Isidoro-Ayza et al., 2017). This is particularly interesting given our sampling method is skin swabbing, which is not the primary tissue that *Rv* colonizes (Miller et al., 2011). However, reproducibility for *Rv* results was lower than both *Bd* and *Pr*, so this finding requires further validation before we can draw any definitive conclusions. Regardless, our study highlights considerable presence of all three pathogens and underscores the need for more amphibian pathogen characterization, monitoring, and mitigation in Florida.

We found slightly higher *Rv* and *Pr* prevalence in metamorphosed (post-metamorphic) individuals compared to tadpoles (pre-metamorphic), despite previously published studies that typically found higher prevalence in pre-metamorphic individuals (Fig. 2D; Cook, 2008; Greer et al., 2005; Karwacki et al., 2018; Miller et al., 2008; Robert et al., 2005). While life stage differences were not significant for either *Rv* or *Pr* prevalence, the modeling results indicate that life stage (as well as species and region) is important for explaining *Pr* prevalence and intensity. Particularly given our limited knowledge on worldwide *Pr* infection dynamics across species, regions, and life stages, elucidating *Pr* infection dynamics in metamorphosed frogs across the anuran tree of life should be the focus of future research. In contrast to *Pr*, neither of the best models explaining *Rv* prevalence or intensity, and only a few of 95% confidence set models, included life stage. Because we measured pathogen burden from skin swabs and *Rv* primarily colonizes internal organs (Miller et al., 2011), our dataset is likely not reflective of functional differences in intensity. Nonetheless, the equivalent rates of pathogen detection across life stages suggests that *Rv* may impact metamorphosed frogs more than anticipated. Similar to our findings for *Pr* and *Rv*, prevalence and intensity of *Bd* was slightly higher in post-metamorphic individuals (Figs. 2D and 3D). While this trend was not significant, this pattern is consistent with numerous other studies documenting higher *Bd* occurrence and disease severity in metamorphosed frogs (e.g., Berger et al., 2016; Laurance et al., 1996; Marantelli et al., 2004; Scheele et al., 2015), and aligns with the fact that *Bd* infections are typically restricted to keratinized mouthparts of tadpoles until metamorphosis (Berger et al., 1998; Marantelli et al., 2004). The modeling results also suggest that life stage is an important component of both *Bd* prevalence and intensity. Thus, our historic data are consistent with findings from more recent studies conducted post-worldwide pathogen spread that suggest that *Bd* infection impacts post-metamorphic more than pre-metamorphic anurans (Berger et al., 2016).

We found that pathogen prevalence across species varied for all three pathogens. All of our sampled species had some level of detection of all three pathogens with the lone exception that *R. virgatipes* harbored no *Bd* (and our total sample size for this species was only 28 individuals). Variation in prevalence is unsurprising for *Bd* based on numerous previous studies documenting that infection and susceptibility vary within and among populations and species (e.g., Berger et al., 2016; Savage and Zamudio, 2011; Scheele et al., 2019). Likewise, widespread presence and variation in *Rv* prevalence is also consistent with other studies documenting infections and mortalities across a broad taxonomic range of host species (Duffus et al., 2015; Miller et al., 2011; Smith et al., 2016). *Pr* was the only pathogen with significant pairwise differences among our species comparisons, and surprisingly, *R. sphenocphala* had significantly lower *Pr* prevalence compared to the other species, despite it being one of the most common species documented with *Pr* infections (Davis et al., 2007; Isidoro-Ayza et al., 2017; Karwacki et al., 2018). Of note is the fact that this study represents the first time *R. heckscheri* has been documented with either *Bd* or *Pr*, the first time *R. virgatipes* has been documented with either *Pr* or *Rv*, the first time *R. grylio* has been documented with *Pr*, and the first time *R. okaloosae* has been documented with *Bd*, *Pr*, or *Rv*. The oldest *R. okaloosae* detected with any pathogens is from 1982, however the museum did not have any specimens older than this. Considering the species was only formally described in 1985 and has a small host range (Moler, 1985), it is unsurprising there were so few specimens present in the collection. Of particular concern is that *Bd* can rapidly spread across populations once

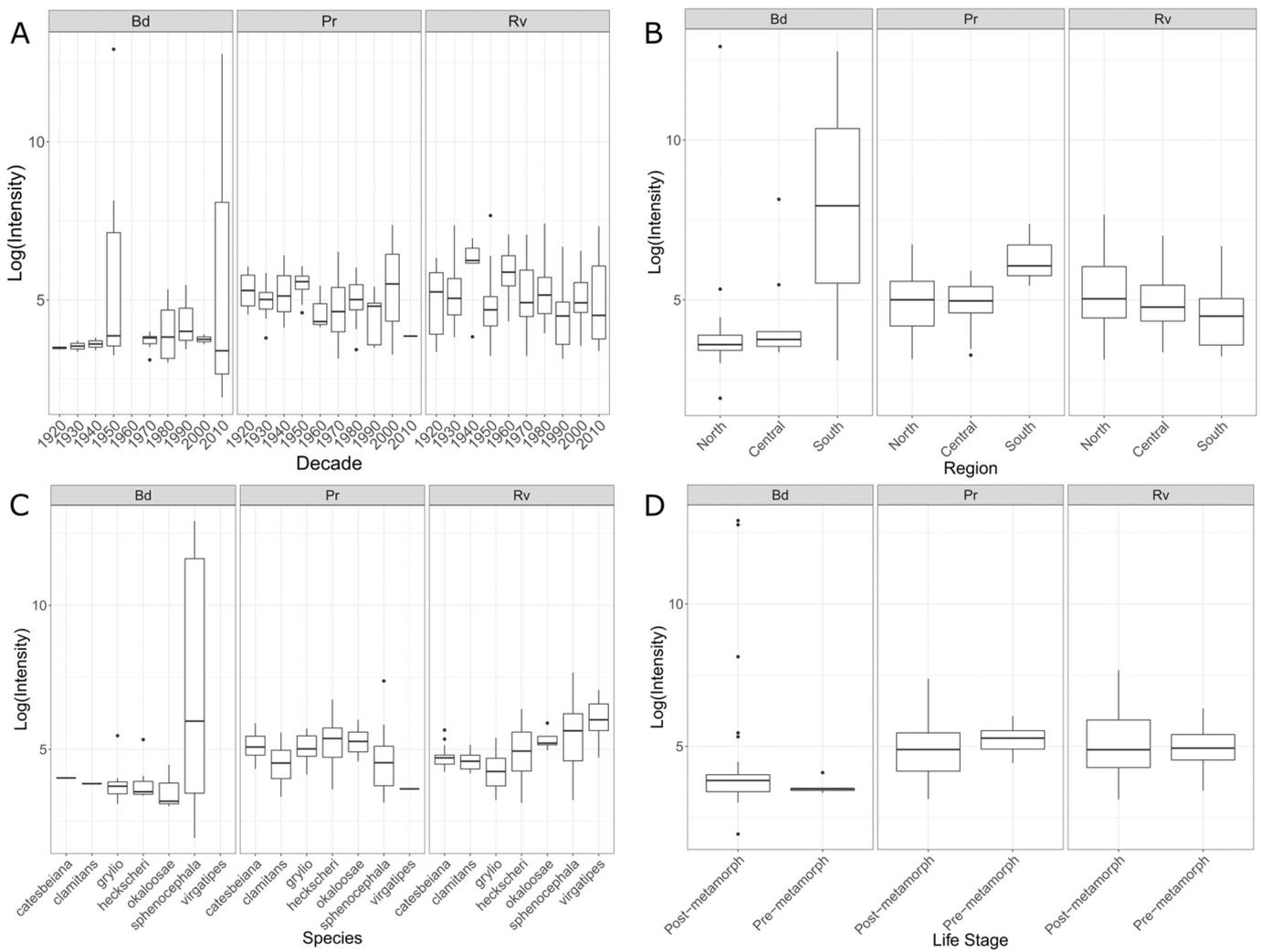


Fig. 3. Box plots of the pathogen detection intensity of *Bd*, *Pr*, and *Rv* compared across A) decades, B) regions of Florida, C) species, and D) life stages.

infections begin and could lead to population extinctions within a few years (Lips et al., 2006; Vredenburg et al., 2010). As *R. okaloosae* is rare and a species of special concern (Moler, 1985), this information helps inform land and wildlife managers about disease concerns within and among populations. Our documentation of all three pathogens in this threatened species highlights how museum specimens offer critical insights about host susceptibility for species that researchers may be unable to readily sample in the field, or unable to obtain permits to capture any for further study.

The seven species examined in our study vary in their current and historical distributions, conservation status, and disease susceptibility. *Rana sphenoccephala* and *R. grylio* are the first and second most abundant frogs in Florida, respectively, and neither species is threatened across their ranges throughout the southeastern U.S. (Ashton and Ashton, 1988; Bartlett and Bartlett, 1999). Similarly, *R. catesbeiana* remain abundant throughout their native U.S. distribution and are successful invaders globally (Ficetola et al., 2007), and *R. clamitans* are not declining or threatened anywhere except for Kansas, where they are listed as threatened (Collins, 1993). *Bd*, *Rv* and *Pr* have each been documented in *R. catesbeiana* (Brunner et al., 2019; Eskew et al., 2015; Isidoro-Ayza et al., 2017), *R. clamitans* (Ouellet et al., 2005), and *R. sphenoccephala* (Davis et al., 2007) and *Pr* has been documented in *R. grylio* (Karwacki et al., 2018). Similarly, we detected all three pathogens in all four of these species in our Florida specimens. Cumulatively, we can therefore say that *R. sphenoccephala*, *R. grylio*, *R. catesbeiana*, and *R. clamitans* have remained relatively stable despite many decades of

pathogen presence. In contrast to these four abundant and widespread species, *R. virgatipes* and *R. heckschleri* have patchy distributions that are poorly characterized (Ashton and Ashton, 1988), and *R. heckschleri* may be extirpated from North Carolina where it has not been seen since 1975 (Beane, 1998). Finally, *R. okaloosae* is endemic to Florida and is considered a species of special concern due to its severely narrow range in the northwestern region of the state and small estimated population sizes (Moler, 1985; Neto et al., 2014). These latter three species are the most likely candidates for historical declines due to disease, but we know extremely little about their historical demographics and pathogen susceptibilities. No previous studies have investigated *Bd*, *Rv* or *Pr* in *R. virgatipes* or *R. okaloosae*, and our only disease knowledge of *R. heckschleri* is a single study documenting *Rv* mortality in wild populations (Miller et al., 2011). Thus, our detection of all three pathogens in *R. okaloosae* and *R. heckschleri*, and of *Rv* or *Pr* in *R. virgatipes*, provide critical documentation that these threatened species may have been infected with most or all three pathogens for many decades and that disease may play a role in their declines. In particular, our finding that *R. okaloosae* had the highest observed prevalence of *Bd* and *Pr* compared to all other species we tested (Fig. 2C) highlights the possibility that disease contributes to the threats faced by this endemic species.

To our knowledge, this study represents the first documentation of tri-detection in an individual with *Bd*, *Pr*, and *Rv*. *Bd* suppresses host immunity (Fites et al., 2013), and could make it easier for co- or tri-infections to occur (Longo et al., 2019). Consistent with this hypothesis, multiple studies have found *Bd* and *Rv* co-infecting individuals (e.g.,

Love et al., 2016; Stutz et al., 2018; Watters et al., 2018; Whitfield et al., 2013), including studies showing that *Bd* presence increases the likelihood of *Rv* infection and vice versa (Love et al., 2016; Miller et al., 2008; Stutz et al., 2018; Watters et al., 2018). One previous study found *Pr* and *Rv* co-infecting *R. catesbeiana* individuals (Landsberg et al., 2013), a species that we found positive for multiple *Pr-Rv* co-detections. Similarly, we found that *Rv* was involved in the two most common co-detections (*Pr-Rv* and *Rv-Bd*) in our samples. However, *Pr-Rv* co-detections occurred significantly more frequently than *Rv-Bd*, suggesting that *Pr* may play an important role in amphibian pathogen co-infections that may be under-reported given that few studies to date have characterized *Pr* (Chambouvet et al., 2015; Isidoro-Ayza et al., 2017; Karwacki et al., 2018). Given the number of different pathogen infections within an individual is positively correlated with mortality (Johnson and Hoverman, 2012; Whitfield et al., 2013), and that many locations with these species also tend to contain multiple micro- and macro-parasites capable of infecting a host (Stutz et al., 2018), the extent of multi-infection with these three pathogens warrants considerable further study.

As with all studies of ancient DNA and preserved specimens, our findings have several important caveats. In particular, unequal sampling across species, decades, regions, and life stages due to limitations of the museum database decreases the statistical power of our modeling approach, particularly the binomial models used to explain pathogen prevalence. Despite these limitations, our model results present useful hypotheses and suggestions for avenues of future research, particularly for *Pr* disease ecology dynamics. Taken with our chi-squared results, which are robust to differences in sample sizes, we have identified several major variables impacting prevalence and intensity of *Bd*, *Pr*, and *Rv*. Importantly, we confirmed *Bd* and *Pr* pathogen presence within our specimens using Sanger sequencing. Given that no *Rv*-positive samples were successfully sequenced, and that repeatability was only 50%, some uncertainty remains regarding whether the qPCR amplifications represent true *Rv* positives. However, the high *Rv* pathogen intensities recovered from numerous specimens using a sensitive and specific TaqMan qPCR assay suggests that these pathogen detections are real, and that the lack of sequencing success and inconsistent qPCR amplifications result from degraded museum DNA template rather than false positives. Successful sequencing of *Bd* and *Pr* barcoding regions from museum specimens revealed no unique genetic variation in samples from the 1970s to 2018 for *Bd*, nor from the 1920s to 2018 for *Pr* (Figs. S1 and S2). Given that the sequenced regions represent short, conserved loci ideal for qPCR, it is unsurprising that no unique genetic variants were detected. Future studies should focus on increasing sample sizes across species and using genome-wide genetic analysis using short-read technology in order to characterize pathogen evolution over the past century and beyond. While older and formalin-preserved DNA specimens are often difficult to work with (Cooper and Poinar, 2000; Schander and Halanych, 2003), our study adds to the body of literature demonstrating the inherent value of data generated from museum specimens. Our pathogen prevalence and intensity data represent conservative estimates because of our stringent requirements for determining qPCR positives, and because sampling from time series specimens tends to increase the likelihood that mass mortality events are missed (Burrowes and De la Riva, 2017b) given the incompleteness of these records (Wandeler et al., 2007). Collections must also be continuously augmented for robust research to continue (Burrowes and De la Riva, 2017b; Lips, 2011) partly because specimens collected prior to extinction events or massive population losses could later lead to confirmations or new discoveries of their causes (Burrowes and De la Riva, 2017b; Carvalho et al., 2017; Puschendorf et al., 2006; Talley et al., 2015). For example, specimens collected in 1986 in Monteverde, Costa Rica prior to a widespread amphibian extinction event later confirmed the presence of *Bd*, implicating the disease as a contributing factor (Puschendorf et al., 2006; Wake and Vredenburg, 2008). Likewise, we were only able to detect significant spikes of *Pr* and *Rv* in the 1920s and

1930s due to the numerous *Rana* specimens collected in Florida in the early 20th century before amphibian disease was a recognized concern. An effective way to ensure important pathogen data is not missed is to include a large number of specimens whenever possible, and to use repeated sampling and specific TaqMan qPCR protocols, as we have done here.

Overall, our results expand the spatial and temporal range of detection of three global pathogens in frog populations from the southeastern U.S. far beyond what has previously been recognized, including 70 more years of *Pr* presence than previously documented anywhere in the world (Chambouvet et al., 2015; Isidoro-Ayza et al., 2017; Karwacki et al., 2018). These data provide critical knowledge on the pathogen landscape experienced by Florida frog populations over the last century, informing conservationists and disease ecologists about whether newly documented infections and mortality events likely represent endemic versus epizootic processes. Importantly, this knowledge will also enable wildlife managers and researchers to predict when, where, and in which species pathogen infections are likely to be most severe (Miller et al., 2018), informing conservation activities ranging from translocation efforts to controlled burns.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank the Florida Museum of Natural History for the generous use of their collections. In particular, we would like to thank curators Dr. Coleman Sheehy and Dr. David Blackburn for assistance in navigating the collections and giving permission to sample so extensively. We would like to thank University of Central Florida (UCF) undergraduates Eleni Triantafyllopoulou and Cassandra Saria and graduate student Veronica Urgiles for assistance in sampling specimens, and undergraduate Hannah Shell in assisting with DNA extractions and qPCR analyses. We would also like to thank graduate student Biplendu Das for assistance with statistical analyses. This work was funded in part by the UCF Office of Undergraduate Research funding to E.E.K.

CRediT authorship contribution statement

Emily Karwacki: Conceptualization, Methodology, Data collection, Data analysis, Writing – Original draft preparation **Katherine Martin:** Data curation, Data analysis, Writing – Reviewing and Editing **Anna Savage:** Conceptualization, Writing– Reviewing and Editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocon.2021.109088>.

References

- Adams, A.J., LaBonte, J.P., Ball, M.L., Richards-Hrdlicka, K.L., Toothman, M.H., Briggs, C.J., 2015. DNA extraction method affects the detection of a fungal pathogen in formalin-fixed specimens using qPCR. *PLoS One* 10. <https://doi.org/10.1371/journal.pone.0135389>.
- Adams, A.J., Pessier, A.P., Briggs, C.J., 2017. Rapid extirpation of a North American frog coincides with an increase in fungal pathogen prevalence: historical analysis and implications for reintroduction. *Ecol. Evol.* 7, 10216–10232. <https://doi.org/10.1002/ece3.3468>.
- Allender, M.C., Bunick, D., Mitchell, M.A., 2013. Development and validation of TaqMan quantitative PCR for detection of frog virus 3-like virus in eastern box turtles (*Terrapene carolina carolina*). *J. Virol. Methods* 188, 121–125. <https://doi.org/10.1016/j.jviromet.2012.12.012>.
- Alroy, J., 2015. Current extinction rates of reptiles and amphibians. *Proc. Natl. Acad. Sci.* <https://doi.org/10.1073/pnas.1508681112>.

- Ashton, Ashton, P.S., 1988. Handbook of Reptiles and Amphibians of Florida. Part 3. Amphibians, Windward Books.
- Bartlett, R.D., Bartlett, P.P., 1999. A Field Guide to Florida Reptiles and Amphibians. Gulf Publishing Company.
- Beane, J.C., 1998. Status of the river frog, *Rana heckscheri* (Anura: Ranidae). In: North Carolina. Brimleyana.
- Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G., Parkes, H., 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci.* <https://doi.org/10.1073/pnas.95.15.9031>.
- Berger, L., Roberts, A.A., Voyles, J., Longcore, J.E., Murray, K.A., Skerratt, L.F., 2016. History and recent progress on chytridiomycosis in amphibians. *Fungal Ecol.* 19, 89–99. <https://doi.org/10.1016/j.funeco.2015.09.007>.
- Bolker, B., Core Team, R., 2017. *lme4: Tools for general maximum likelihood estimation*. R package version 1.0. 20. In: R Foundation for Statistical Computing.
- Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T., Hyatt, A.D., 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Org.* 60, 141–148.
- Brunner, J.L., Olson, A.D., Rice, J.G., Meiners, S.E., Le Sage, M.J., Cundiff, J.A., Goldberg, C.S., Pessier, A.P., 2019. Ranavirus infection dynamics and shedding in American bullfrogs: consequences for spread and detection in trade. *Dis. Aquat. Org.* 135 <https://doi.org/10.3354/dao03387>.
- Burrowes, P.A., De la Riva, I., 2017a. Unraveling the historical prevalence of the invasive chytrid fungus in the Bolivian Andes: implications in recent amphibian declines. *Biol. Invasions* 19, 1781–1794. <https://doi.org/10.1007/s10530-017-1390-8>.
- Burrowes, P.A., De la Riva, I., 2017b. Detection of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in museum specimens of andean aquatic birds: implications for pathogen dispersal. *J. Wildl. Dis.* 53, 349–355. <https://doi.org/10.7589/2016-04-074>.
- Campbell, I., 2007. Chi-squared and Fisher-Irwin tests of two-by-two tables with small sample recommendations. *Stat. Med.* 26, 3661–3675.
- Carvalho, T., Guilherme Becker, C., Toledo, L.F., 2017. Historical amphibian declines and extinctions in Brazil linked to chytridiomycosis. *Proc. R. Soc. B Biol. Sci.* 284, 20162254 <https://doi.org/10.1098/rspb.2016.2254>.
- Chambouvet, A., Gower, D.J., Jirků, M., Yabsley, M.J., Davis, A.K., Leonard, G., Maguire, F., Doherty-Bone, T.M., Bittencourt-Silva, G.B., Wilkinson, M., Richards, T.A., 2015. Cryptic infection of a broad taxonomic and geographic diversity of tadpoles by *Perkinsea* protists. *Proc. Natl. Acad. Sci.* <https://doi.org/10.1073/pnas.1500163112>.
- Cheng, T.L., Rovito, S.M., Wake, D.B., Vredenburg, V.T., 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proc. Natl. Acad. Sci.* <https://doi.org/10.1073/pnas.1105538108>.
- Collins, J.T., 1993. *Amphibians and Reptiles in Kansas*, 3rd ed. University of Kansas, Lawrence.
- Cook, J.O., 2008. *Transmission and Occurrence of Dermomycoides Sp. in Rana Sevosa and Other Ranids in the North and Central Gulf of Mexico States*. University of Southern Mississippi, Hattiesburg, MS.
- Cooper, A., Poinar, H., 2000. Ancient DNA do it right or not at all. *Science* (80-). 1139.
- Cunningham, A.A., 2018. *Infectious disease threats to amphibian conservation*. *Glas. Nat.* 27.
- Daszak, P., Berger, L., Cunningham, A.A., Hyatt, A.D., Green, D.E., Speare, R., 1999. Emerging infectious diseases and amphibian population declines. *Emerg. Infect. Dis.* 5, 735.
- Davis, A.K., Yabsley, M.J., Kevin Keel, M., Maerz, J.C., 2007. Discovery of a novel alveolate pathogen affecting southern leopard frogs in Georgia: description of the disease and host effects. *Ecohealth* 4, 310–317. <https://doi.org/10.1007/s10393-007-0115-3>.
- Duffus, A.L., Waltzek, T.B., Stohr, A.C., Allender, M.C., Gotesman, M., Whittington, R.J., Hick, P., Hines, M.K., Marschang, R.E., 2015. Distribution and host range of Ranaviruses. In: Gray, M., Chinchar, V. (Eds.), *Ranaviruses*. Springer, Cham, pp. 9–57. https://doi.org/10.1007/978-3-319-13755-1_2.
- Eskew, E.A., Worth, S.J., Foley, J.E., Todd, B.D., 2015. American Bullfrogs (*Lithobates catesbeianus*) Resist Infection by Multiple Isolates of *Batrachochytrium dendrobatidis*, Including One Implicated in Wild Mass Mortality. *Ecohealth*. doi:<https://doi.org/10.1007/s10393-015-1035-2>.
- Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., Cunningham, A.A., Weldon, C., du Preez, L.H., Anderson, L., Pond, S.L.K., Shahr-Golan, R., Henk, D.A., Fisher, M.C., 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proc. Natl. Acad. Sci.* 108, 18732–18736. <https://doi.org/10.1073/pnas.1111915108>.
- Ficetola, G.F., Thuiller, W., Miaud, C., 2007. Prediction and validation of the potential global distribution of a problematic alien invasive species - the American bullfrog. *Divers. Distrib.* 13 <https://doi.org/10.1111/j.1472-4642.2007.00377.x>.
- Fisher, M.C., Garner, T.W.J., Walker, S.F., 2009. Global emergence of *Batrachochytrium dendrobatidis* and amphibian Chytridiomycosis in Space, Time, and Host. *Annu. Rev. Microbiol.* 63, 291–310. <https://doi.org/10.1146/annurev.micro.091208.073435>.
- Fites, J.S., Ramsey, J.P., Holden, W.M., Collier, S.P., Sutherland, D.M., Reinert, L.K., Gayek, A.S., Dermody, T.S., Aune, T.M., Oswald-Richter, K., Rollins-Smith, L.A., 2013. The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. *Science* (80-). 342, 366–369. doi:<https://doi.org/10.1126/science.1243316>.
- Fovell, R.G., Fovell, M.Y.C., 1993. Climate zones of the conterminous United States defined using cluster analysis. *J. Clim.* 6 [https://doi.org/10.1175/1520-0442\(1993\)006<2103:CZOTCU>2.0.CO;2](https://doi.org/10.1175/1520-0442(1993)006<2103:CZOTCU>2.0.CO;2).
- Gantress, J., Maniero, G.D., Cohen, N., Robert, J., 2003. Development and characterization of a model system to study amphibian immune responses to iridoviruses. *Virology* 311, 254–262. [https://doi.org/10.1016/S0042-6822\(03\)00151-X](https://doi.org/10.1016/S0042-6822(03)00151-X).
- Gray, M.J., Miller, D.L., Hoverman, J.T., 2009. Ecology and pathology of amphibian ranaviruses. *Dis. Aquat. Org.* <https://doi.org/10.3354/dao02138>.
- Green, E.D., Dodd, K.C., 2007. Presence of amphibian chytrid fungus *Batrachochytrium dendrobatidis* and other amphibian pathogens at warm-water fish hatcheries in southeastern North America. *Herpetol. Conserv. Biol.* 2, 43–47.
- Green, D.E., Converse, K.A., Schrader, A.K., 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996–2001, in: *Annals of the New York Academy of Sciences*. doi:<https://doi.org/10.1111/j.1749-6632.2002.tb04400.x>.
- Green, D.E., Feldman, S.H., Wimsatt, J., 2003. Emergence of a *Perkinsus*-like agent in anuran liver during die-offs of local populations: PCR detection and phylogenetic characterization, in: *Joint Conference - American Association of Zoo Veterinarians*. Minneapolis, MN, pp. 120–121.
- Greer, A.L., Berrill, M., Wilson, P.J., 2005. Five amphibian mortality events associated with ranavirus infection in south Central Ontario, Canada. *Dis. Aquat. Org.* 67, 9–14.
- Heberling, J.M., Prather, L.A., Tonsor, S.J., 2019. The changing uses of herbarium data in an era of global change: an overview using automated content analysis. *Bioscience*. <https://doi.org/10.1093/biosci/biz094>.
- Hornor, A.A., Hoffman, E.A., Tye, M.R., Hether, T.D., Savage, A.E., 2017. Cryptic chytridiomycosis linked to climate and genetic variation in amphibian populations of the southeastern United States. *PLoS One*. <https://doi.org/10.1371/journal.pone.0175843>.
- Huss, M., Huntley, L., Vredenburg, V., Green, S., Johns, J., 2014. Prevalence of *Batrachochytrium dendrobatidis* in 120 archived specimens of *Lithobates catesbeianus* (American bullfrog) collected in California, 1924–2007. *Ecohealth* 10, 339–343. <https://doi.org/10.1007/s10393-013-0895-6>.
- Hyatt, A.D., Boyle, D.G., Olsen, V., Boyle, D.B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., Phillott, R., Campbell, R., Marantelli, G., Gleason, F., Colling, A., 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Org.* <https://doi.org/10.3354/dao073175>.
- Isidoro-Ayza, M., Lorch, J.M., Grear, D.A., Winzeler, M., Calhoun, D.L., Barichivich, W.J., 2017. Pathogenic lineage of *Perkinsea* associated with mass mortality of frogs across the United States. *Sci. Rep.* 7, 10288. <https://doi.org/10.1038/s41598-017-10456-1>.
- Jancovich, J.K., Davidson, E.W., Parameswaran, N., Mao, J., Chinchar, V.G., Collins, J.P., Jacobs, B.L., Storfer, A., 2005. Evidence for emergence of an amphibian iridoviral disease because of human-enhanced spread. *Mol. Ecol.* 14, 213–224. <https://doi.org/10.1111/j.1365-294X.2004.02387.x>.
- Johnson, P.T.J., Hoverman, J.T., 2012. Parasite diversity and coinfection determine pathogen infection success and host fitness. *Proc. Natl. Acad. Sci.* <https://doi.org/10.1073/pnas.1201790109>.
- Karwacki, E.E., Atkinson, M.S., Ossiboff, R.J., Savage, A.E., 2018. Novel quantitative PCR assay specific for the emerging *Perkinsea* amphibian pathogen reveals seasonal infection dynamics. *Dis. Aquat. Org.* 129, 85–98.
- Kiesecker, J.M., Blaustein, A.R., Belden, L.K., 2001. Complex causes of amphibian population declines. *Nature* 410, 681–684. <https://doi.org/10.1038/35070552>.
- Koo, K.-S., Park, I.-K., Moon, K.-Y., Lee, J.-G., Park, D., 2017. PCR detection of Ranavirus from dead *Kaloula borealis* and sick *Hyla japonica* tadpoles in the wild. *Korean Journal of Herpetology*. 8, 10–14.
- La Marca, E., Lips, K.R., Lötters, S., Puschendorf, R., Ibáñez, R., Rueda-Almonacid, J.V., Schulte, R., Marty, C., Castro, F., Manzani-Puppo, J., García-Pérez, J.E., Bolaños, F., Chaves, G., Pounds, J.A., Toral, E., Young, B.E., 2005. Catastrophic population declines and extinctions in neotropical harlequin frogs (*Bufo* spp.: *Atelopus*). *Biotropica* 37, 190–201. <https://doi.org/10.1111/j.1744-7429.2005.00026.x>.
- Landsberg, J.H., Kiryu, Y., Tabuchi, M., Waltzek, T.B., Enge, K.M., Reintjes-Tolen, S., Preston, A., Pessier, A.P., 2013. Co-infection by alveolate parasites and frog virus 3-like ranavirus during an amphibian larval mortality event in Florida, USA. *Dis. Aquat. Org.* 105, 89–99. <https://doi.org/10.3354/dao02625>.
- Laurance, W.F., McDonald, K.R., Speare, R., 1996. *Epidemic Disease and the Catastrophic Decline of Australian Rain Forest Frogs*, Biology.
- Lips, K.R., 2011. Museum collections: mining the past to manage the future. *Proc. Natl. Acad. Sci.* 108, 9323–9324. <https://doi.org/10.1073/pnas.1107246108>.
- Lips, K.R., Brem, F., Brenes, R., Reeve, J.D., Alford, R.A., Voyles, J., Carey, C., Livo, L., Pessier, A.P., Collins, J.P., Wake, D.B., 2006. Emerging Infectious Disease and the Loss of Biodiversity in a Neotropical Amphibian Community. *PNAS February*.
- Longcore, J.E., Pessier, A.P., Nichols, D.K., 1999. *Batrachochytrium Dendrobatidis* gen. et sp. nov., a Chytrid Pathogenic to Amphibians. *Mycologia* 219–227. doi:<https://doi.org/10.2307/3761366>.
- Longo, A.V., Fleischer, R.C., Lips, K.R., 2019. Double trouble: co-infections of chytrid fungi will severely impact widely distributed newts. *Biol. Invasions*. <https://doi.org/10.1007/s10530-019-01973-3>.
- Love, C.N., Winzeler, M.E., Beasley, R., Scott, D.E., Nunziata, S.O., Lance, S.L., 2016. Patterns of amphibian infection prevalence across wetlands on the Savannah River Site, South Carolina, USA. *Dis. Aquat. Org.* 121, 1–14. doi:<https://doi.org/10.3354/dao03039>.
- Marantelli, G., Berger, L., Speare, R., Keegan, L., 2004. Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pacific Conserv. Biol.* 10, 173–179.

- Meshaka, W.E. J., 2001. The Cuban treefrog in Florida: life history of a successful colonizing species. *Univ. Press Florida*. doi:<https://doi.org/10.5860/choice.39-5819>.
- Miaud, C., Pozet, F., Gaudin, N.C.G., Martel, A., Pasmans, F., Labrut, S., 2016. Ranavirus causes mass die-offs of alpine amphibians in the southwestern Alps, France. *J. Wildl. Dis.* 52, 242–252. <https://doi.org/10.7589/2015-05-113>.
- Miller, D.L., Brookins, M.A., Cook, J., Whittington, L., Baldwin, C.A., 2008. Concurrent infection with Ranavirus, *Batrachochytrium dendrobatidis*, and *Aeromonas* in a Captive Anuran Colony. *Source J. Zoo Wildl. Med.* 39, 445–449. <https://doi.org/10.1638/2008-0012.1>.
- Miller, D., Gray, M., Storfer, A., 2011. Ecopathology of ranaviruses infecting amphibians. *Viruses* 3, 2351–2373. <https://doi.org/10.3390/v3112351>.
- Miller, C.A., Canis Tasse Taboue, G., Ekane, M.M.P., Robak, M., Sesink Clew, P.R., Richards-Zawacki, C., Fokam, E.B., Fuashi, N.A., Anthony, N.M., 2018. Distribution modeling and lineage diversity of the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) in a central African amphibian hotspot. *PLoS One* 13. <https://doi.org/10.1371/journal.pone.0199288>.
- Moler, P.E., 1985. A new species of frog (Ranidae): Rana from Northwestern Florida. *Copeia* 1985, 379–383. <https://doi.org/10.2307/1444847>.
- Moriarty, J.J., 2017. *Amphibian and Reptile Diversity and Distribution in the United States*. FrogWatch.
- Mutnale, M.C., Anand, S., Eluvathingal, L.M., Roy, J.K., Reddy, G.S., Vasudevan, K., 2018. Zoogenic frog pathogen *Batrachochytrium dendrobatidis* in Asian tropics reveals high ITS haplotype diversity and low prevalence. *Sci. Rep.* 8, 1–11. <https://doi.org/10.1038/s41598-018-28304-1>.
- Neto, J.G.D.S., Gorman, T.A., Bishop, D.C., Haas, C.A., 2014. Population demographics of the Florida Bog Frog (*Lithobates okaloosae*). *Southeast. Nat.* 13. <https://doi.org/10.1656/058.013.0113>.
- North, A.C., Hodgson, D.J., Price, S.J., Griffiths, A.G.F., 2015. Anthropogenic and ecological drivers of amphibian disease (ranaviruses). *PLoS One* 10. <https://doi.org/10.1371/journal.pone.0127037>.
- O'hlanon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A., Kosch, T.A., Murray, K.A., Brankovics, B., Fumagalli, M., Martin, M.D., Wales, N., Alvarado-Rybak, M., Bates, K.A., Berger, L., Böll, S., Brookes, L., Clare, F., Courtois, E.A., Cunningham, A.A., Doherty-Bone, T.M., Ghosh, P., Gower, D.J., Hintz, W.E., Höglund, J., Jenkinson, T.S., Lin, C.-F., Laurila, A., Loyau, A., Martel, A., Meurling, S., Miaud, C., Minting, P., Pasmans, F., Schmeidler, D.S., Schmidt, B.R., Shelton, J.M.G., Skerratt, L.F., Smith, F., Soto-Azat, C., Spagnoletti, M., Tessa, G., Toledo, L.F., Valenzuela-Sánchez, A., Verster, R., Vörös, J., Webb, R.J., Wierzbicki, C., Wombwell, E., Zamudio, K.R., Aanensen, D.M., James, T.Y., Thomas, M., Gilbert, P., Weldon, C., Bosch, J., Balloux, F., Trenton, J., Garner, W.J., Matthew, J., Fisher, C., 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science* (80-). 360, 612–627.
- Ouellet, M., Mikaelian, I., Pauli, B.D., Rodrigue, J., Green, D.M., 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. *Conserv. Biol.* 19, 1431–1440. <https://doi.org/10.1111/j.1523-1739.2005.00108.x>.
- Puschendorf, R., Bolaños, F., Chaves, G., 2006. The amphibian chytrid fungus along an altitudinal transect before the first reported declines in Costa Rica. *Biol. Conserv.* 132, 136–142. <https://doi.org/10.1016/j.biocon.2006.03.010>.
- R Core Team, 2019. A language and environment for statistical computing.
- Richards-Hrdlicka, K.L., 2012. Extracting the amphibian chytrid fungus from formalin-fixed specimens. *Methods Ecol. Evol.* 3, 842–849. <https://doi.org/10.1111/j.2041-210X.2012.00228.x>.
- Rijks, J.M., Saucedo, B., Spitzen-Van Der Sluijs, A., Wilkie, G.S., Van Asten, A.J.A.M., Van Broek, J. Den, Boonyarittichai, R., Stege, M., Van Sterren, F. Der, Martel, A., Pasmans, F., Hughes, J., Gröne, A., Van Beurden, S.J., Kik, M.J.L., 2016. Investigation of amphibian mortality events in wildlife reveals an on-going Ranavirus epidemic in the north of the Netherlands. *PLoS One* 11. <https://doi.org/10.1371/journal.pone.0157473>.
- Rizkalla, C.E., 2009. First reported detection of *Batrachochytrium dendrobatidis* in Florida, USA. *Herpetol. Rev.* 40, 189–190.
- Robert, J., Morales, H., Buck, W., Cohen, N., Marr, S., Gantress, J., 2005. Adaptive immunity and histopathology in frog virus 3-infected *Xenopus*. *Virology*. <https://doi.org/10.1016/j.virol.2004.12.012>.
- Rothermel, B.B., Walls, S.C., Mitchell, J.C., Dodd, C.K., Irwin, L.K., Green, D.E., Vazquez, V.M., Petranka, J.W., Stevenson, D.J., 2008. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. *Dis. Aquat. Org.* 82, 3–18. <https://doi.org/10.3354/dao01974>.
- Savage, A.E., Zamudio, K.R., 2011. MHC genotypes associate with resistance to a frog-killing fungus. *Proc. Natl. Acad. Sci.* 108, 16705–16710. <https://doi.org/10.1073/pnas.1106893108>.
- Schander, C., Halanaych, K.M., 2003. DNA, PCR and formalinized animal tissue - a short review and protocols. *Org. Divers. Evol.* 3, 195–205.
- Scheele, B.C., Hunter, D.A., Skerratt, L.F., Brannelly, L.A., Driscoll, D.A., 2015. Low impact of chytridiomycosis on frog recruitment enables persistence in refuges despite high adult mortality. *Biol. Conserv.* 182, 36–43. <https://doi.org/10.1016/j.biocon.2014.11.032>.
- Scheele, B.C., Pasmans, F., Skerratt, L.F., Berger, L., Martel, A., Beukema, W., Acevedo, A.A., Burrows, P.A., Carvalho, T., Catenazzi, A., De la Riva, I., Fisher, M.C., Flechas, S. V., Foster, C.N., Frias-Álvarez, P., J. Garner, T.W., Gratwicke, B., Guayasamin, J.M., Hirschfeld, M., Kolby, J.E., Kosch, T.A., La Marca, E., Lindenmayer, D.B., Lips, K.R., Longo, A. V., Maneyro, R., McDonald, C.A., Mendelson III, J., Palacios-Rodríguez, P., Parra-Olea, G., Richards-Zawacki, C.L., Rödel, M.-O., Rovito, S.M., Soto-Azat, C., Felipe Toledo, L., Voyles, J., Weldon, C., Whitfield, S.M., Wilkinson, M., Zamudio, K. R., Canessa, S., 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science* (80-). 363, 1459–1463.
- Schloegel, L.M., Picco, A.M., Kilpatrick, A.M., Davies, A.J., Hyatt, A.D., Daszak, P., 2009. Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*). *Biol. Conserv.* 142, 1420–1426. <https://doi.org/10.1016/j.biocon.2009.02.007>.
- Schloegel, L.M., Toledo, L.F., Longcore, J.E., Greenspan, S.E., Vieira, C.A., Lee, M., Zhao, S., Wangen, C., Ferreira, C.M., Hipolito, M., Davies, A.J., Cuomo, C.A., Daszak, P., James, T.Y., 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Mol. Ecol.* 21, 5162–5177. <https://doi.org/10.1111/j.1365-294X.2012.05710.x>.
- Schoch, C.L., Robbertse, B., Robert, V., Vu, D., Cardinali, G., Irinyi, L., Meyer, W., Nilsson, R.H., Hughes, K., Miller, A.N., Kirk, P.M., Abarenkov, K., Aime, M.C., Ariyawansa, H.A., Bidartondo, M., Boekhout, T., Buyck, B., Cai, Q., Chen, J., Crespo, A., Crous, P.W., Damm, U., De Beer, Z.W., Dentinger, B.T.M., Divakar, P.K., Duenas, M., Feau, N., Fliegerova, K., García, M.A., Ge, Z.W., Griffith, G.W., Groenewald, J.Z., Groenewald, M., Grube, M., Gryzenhout, M., Gueidan, C., Guo, L., Hambleton, S., Hamelin, R., Hansen, K., Hofstetter, V., Hong, S.B., Houbraeken, J., Hyde, K.D., Inderbitzin, P., Johnston, P.R., Karunarathna, S.C., Kõljalg, U., Kovács, G.M., Kraichak, E., Krizsan, K., Kurtzman, C.P., Larsson, K.H., Leavitt, S., Letcher, P.M., Liimatainen, K., Liu, J.K., Lodge, D.J., Luangsa-Ard, J.J., Lumbsch, H. T., Maharachchikumbura, S.S.N., Manamgoda, D., Martín, M.P., Minnis, A.M., Moncalvo, J.M., Mulè, G., Nakasone, K.K., Niskanen, T., Olariaga, I., Papp, T., Petkovits, T., Pino-Bodas, R., Powell, M.J., Raja, H.A., Redecker, D., Sarmiento-Ramirez, J.M., Seifert, K.A., Shrestha, B., Stenroos, S., Stielow, B., Suh, S.O., Tanaka, K., Tedersoo, L., Telleria, M.T., Udayanga, D., Unterseiner, W.A., Uribeondo, J.D., Subbarao, K.V., Vágvölgyi, C., Visagie, C., Voigt, K., Walker, D.M., Weir, B.S., Weiß, M., Wijayawardene, N.N., Wingfield, M.J., Xu, J.P., Yang, Z.L., Zhang, N., Zhuang, W.Y., Federhen, S., 2014. Finding needles in haystacks: Linking scientific names, reference specimens and molecular data for fungi. In: Database 2014. <https://doi.org/10.1093/database/bau061>.
- Seimon, T.A., Ayebare, S., Sekisambu, R., Muhindo, E., Mitamba, G., Greenbaum, E., Menegon, M., Pupin, F., McAlouse, D., Ammazalorso, A., Meirte, D., Lukwago, W., Behangana, M., Seimon, A., Plumtree, A.J., 2015. Assessing the threat of amphibian chytrid fungus in the Albertine Rift: past, present and future. *PLoS One*. <https://doi.org/10.1371/journal.pone.0145841>.
- Skerratt, L.F., Berger, L., Speare, R., Cashins, S., McDonald, K.R., Phillott, A.D., Hines, H. B., Kenyon, N., 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth*. <https://doi.org/10.1007/s10393-007-0093-5>.
- Smith, S.A., Monsen-Collar, K.J., Green, D.E., Niederriter, H.S., Hall, M.L., Terrell, K.A., Gipe, K.D., Urban, C.A., Patterson, C.A., Seigel, R.A., Zarate, B., Kleopfer, J.D., Campbell-Grant, E.H., Driscoll, C.P., 2016. Detecting the Extent of Mortality Events from Ranavirus in Amphibians of the Northeastern U.S.
- Soto-Azat, C., Clarke, B.T., Fisher, M.C., Walker, S.F., Cunningham, A.A., 2009. Non-invasive sampling methods for the detection of *Batrachochytrium dendrobatidis* in archived amphibians. *Dis. Aquat. Org.* 84, 163–166. <https://doi.org/10.3354/dao02029>.
- Stutz, W.E., Blaustein, A.R., Briggs, C.J., Hoverman, J.T., Rohr, J.R., Johnson, P.T.J., 2018. Using multi-response models to investigate pathogen coinfections across scales: insights from emerging diseases of amphibians. *Methods Ecol. Evol.* 9, 1109–1120. <https://doi.org/10.1111/2041-210X.12938>.
- Symonds, M.R., Moussalli, A., 2011. A brief guide to model selection, multimodel inference and model averaging in behavioural ecology using Akaike's information criterion. *Behav. Ecol. Sociobiol.* 65, 13–21.
- Talley, B.L., Muletz, C.R., Vredenburg, V.T., Fleischer, R.C., Lips, K.R., 2015. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). *Biol. Conserv.* 182, 254–261. <https://doi.org/10.1016/j.biocon.2014.12.007>.
- The IUCN Red List of Threatened Species, 2020. [WWW Document], URL. www.iucnredlist.org/.
- Van Rooij, P., Martel, A., Haesebrouck, F., Pasmans, F., 2015. Amphibian chytridiomycosis: a review with focus on fungus-host interactions. *Vet. Res.* 46, 1–22. <https://doi.org/10.1186/s13567-015-0266-0>.
- Voyles, J., Berger, L., Young, S., Speare, R., Webb, R., Warner, J., Rudd, D., Campbell, R., Skerratt, L.F., 2007. Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Dis. Aquat. Org.* 77, 113–118. <https://doi.org/10.3354/dao01838>.
- Vredenburg, V.T., Knapp, R.A., Tunstall, T.S., Briggs, C.J., 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc. Natl. Acad. Sci.* 107, 9689–9694. <https://doi.org/10.1073/pnas.0914111107>.
- Wake, D.B., Vredenburg, V.T., 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc. Natl. Acad. Sci.* 105, 11466–11473.
- Wandeler, P., Hoecq, P.E.A., Keller, L.F., 2007. Back to the future: museum specimens in population genetics. *Trends Ecol. Evol.* 22, 634–642. <https://doi.org/10.1016/j.tree.2007.08.017>.
- Wang, H., Wang, H.N., Regassa, J.F., Wang, X.L., 2018. Independent origin of chytrid fungus in China. *Russ. J. Herpetol.* 25, 299–310. doi:10.30906/1026-2296-2018-25-4-299-310.
- Watters, J.L., Davis, D.R., Yuri, T., Siler, C.D., 2018. Concurrent infection of *Batrachochytrium dendrobatidis* and Ranavirus among native amphibians from northeastern Oklahoma, USA. *J. Aquat. Anim. Health*. <https://doi.org/10.1002/aah.10041>.
- Weldon, C., Du Prees, L.H., Hyatt, A.D., Muller, R., Speare, R., 2004. Origin of the amphibian chytrid fungus. *Emerg. Infect. Dis.* 10, 2100.
- White, M.R., Lips, K.R., Pringle, C.M., Kilham, S.S., Bixby, R.J., Brenes, R., Connelly, S., Colon-Gaud, J.C., Hunte-Brown, M., Huryn, A.D., Montgomery, C., 2006. The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. *Front. Ecol. Environ.* 4, 27–34.

- Whitfield, S.M., Geerdes, E., Chacon, I., Ballester Rodriguez, E., Jimenez, R.R., Donnelly, M.A., Kerby, J.L., 2013. Infection and co-infection by the amphibian chytrid fungus and ranavirus in wild Costa Rican frogs. *Dis. Aquat. Org.* 104, 173–178. <https://doi.org/10.3354/dao02598>.
- Youker-Smith, T.E., Boersch-Supan, P.H., Whipps, C.M., Ryan, S.J., 2018. Environmental drivers of Ranavirus in free-living amphibians in constructed ponds. *Ecohealth* 15, 608–618. <https://doi.org/10.1007/s10393-018-1350-5>.
- Zhu, W., Bai, C., Wang, S., Soto-Azat, C., Li, X., Liu, X., Li, Y., 2014. Retrospective survey of museum specimens reveals historically widespread presence of *Batrachochytrium dendrobatidis* in China. *Ecohealth* 11, 241–250. <https://doi.org/10.1007/s10393-013-0894-7>.