Human Impacts on the Prevalence of the Amphibian Infectious Diseases, *Batrachochytrium dendrobatidis* and Ranavirus, in Oklahoma, USA

Jessa L. Watters
Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK 73072

Spencer E. Hall*
Department of Biology, University of Oklahoma, Norman, OK 73069

Cameron D. Siler
Department of Biology, University of Oklahoma, Norman, OK 73069

Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK 73072

Abstract: *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus (RV) are pathogens contributing to the global decline of amphibian populations. Both pathogens can be spread through direct contact between amphibians, through water carrying the infection, the accidental movement of disease particles between waterbodies by cattle, boats, or aquatic recreational equipment, or the intentional movement of infected amphibians used as fishing bait. Amphibians can also experience indirect human-caused effects due to environmental pollutants, including increased stress levels and reduced immunity. We conducted a meta-analysis regarding the effects of human impact on *Bd* and RV pathogen prevalence and infection loads in Oklahoma amphibians, based on field research conducted 2015–2017. Research sites were identified as having minimal, moderate, or high human impact with regard to the degree of land usage for aquatic recreation, grazing, and oil/natural gas. Samples were screened for both *Bd* (gene ITS1) and RV (gene MCP) via published qPCR methodologies; results are reported for both prevalence and infection load (calculated based on qPCR output of mean gene copies multiplied by the dilution factor and extraction volume). We found an average prevalence of 47% for *Bd* and 19.2% for RV infection in amphibians (sample sites pooled), with a trend of increasing prevalence for *Bd* and RV with increasing human interaction. For both pathogens, specimens collected from "moderate" sites had the highest infection loads. We advise land managers overseeing the public use of Oklahoma lands to share educational material regarding amphibian infectious disease, to prevent future spread.

Introduction

Global amphibian declines have been at the forefront of herpetological research for over three decades, yet no single threat has been pinpointed as the primary cause. Instead, amphibian declines have been linked to synergistic effects between several threats, including habitat loss and modification, environmental pollutants, over-exploitation for food and the pet trade, invasive species, climate change, and infectious diseases (McMenamin et al., 2008; Grant et al., 2020; Ford et al., 2020). The interplay of all of these largely anthropogenic factors has been difficult to tease apart, despite extensive research (Green et al., 2020) and making substantial

*Current address: Hudson College of Public Health, University of Oklahoma, Oklahoma City, OK

headway to decrease amphibian decline will require changes to policy and society as well (Beebee and Griffiths, 2005; Ford et al., 2020). Many researchers consider the modern spread of infectious diseases to be one of the most alarming threats to amphibians, especially as it is exacerbated by climate change and introductions to native amphibian communities (Pounds et al., 2006; Bienentreau and Lesbarreres, 2020). Two diseases now recognized to be major contributors to amphibian decline are chytridiomycosis, often referred to as chytrid and caused by the fungus *Batrachochytrium dendrobatidis* (***Bd***), and a systemic infection caused by strains of ranavirus (RV). Chytridiomycosis is a skin infection that clogs and unravels keratinized amphibian tissue, decreasing the host's ability to control osmotic balance and undergo cutaneous respiration, often leading to higher mortality in amphibian populations (Voyles et al., 2009). Less severe symptoms include lethargy, loss of appetite, and skin sloughing (O'Hanlon et al., 2018); however, these too have negative impacts on populations as they often lead to increased susceptibility to predation (Berger et al., 1998; Han et al., 2011). Keratinized structures in amphibians are reduced in pre-metamorphic individuals, often associated with oral regions only, therefore, they are often less susceptible to widespread *Bd* infection (Berger et al., 1998), though starvation has been known to occur (Venesky et al., 2010).

Like *Bd,* RV-infected individuals experience symptoms of emaciation and lethargy that increase their susceptibility to predation (Harp and Petranka, 2006). Furthermore, RV-infected organisms can experience organ necrosis and hemorrhaging, ultimately leading to death (Gray et al., 2009; Gray and Chinchar, 2015). Tadpoles are particularly vulnerable to RV infection, with mortality rates now shown to increase exponentially with each stage of larval development (Warne et al., 2011). Furthermore, because ranavirus is able to switch hosts easily among several major vertebrate groups, including amphibians, some reptiles, and fish, it raises great concern for those studying infectious diseases on these potentially vulnerable populations (Jancovich et al., 2005; Currylow et al., 2014; Gray and Chinchar, 2015). Amphibians with *Bd* and RV comorbidities are at greatest risk, with cases of coinfections documented previously in wild populations in the tropical Andes, Costa Rica, and Oklahoma, USA (Whitfield et al., 2013; Warne et al., 2016; Watters et al., 2018). For both *Bd* and RV, pathogens may only cause deleterious effects in some species, whereas tolerant species act as carriers that spread the disease to more vulnerable species (Schloegel et al., 2009; Hoverman et al., 2011; Currylow et al., 2014).

Research indicates that both *Bd* and RV have been spread worldwide through the global commercial amphibian trade (Schloegel et al., 2009; O'Hanlon et al., 2018), mostly during a period of time when traders did not use handling methods that would prevent cross-infections (Weldon et al., 2004; Fisher and Garner, 2007; Price et al., 2016). Humans continue to spread both diseases through direct effects and can contribute to disease susceptibility though indirect effects in a number of ways (Gray et al., 2017). First, some interactions such as human aquatic recreational activities and cattle grazing can spread both *Bd* and RV directly (Jancovich et al., 2005; Gray et al., 2007; Greer and Collins 2008; Gray et al., 2017). For example, humans engaging in recreational activities like fishing, hunting, and boating can spread *Bd* and RV by moving from pond to pond without disinfecting equipment (i.e. boats, waders, nets, etc.) that comes into contact with water or mud (Cunningham et al., 2003; Gray et al., 2017; Casais et al., 2019), or even moving infected individuals between locations by using them as fishing bait (Jancovich et al., 2005; Picco and Collins, 2008). Indirect human-mediated stressors have also been shown to increase amphibian susceptibility to disease through immune suppression, such as the use of pesticides, human road traffic, modification of habitats, and oil and natural gas extraction (Kerby and Storfer, 2009; Kerby et al., 2011; Brittingham et al., 2014; Guo et al., 2018; Robert et al., 2019; Bienentreau and Lesbarreres, 2020). Furthermore, significant habitat modification can result in increased amphibian densities in remaining, fragmented habitats, leading to higher rates of pathogen transmission (Bienentreau and

Lesbarreres, 2020). Additionally, the presence of cattle grazing in the vicinity of waterbodies also provides both direct and indirect effects through transfer microbes contained in sediments between locations (on hooves), degradation of water quality, and decreases in vegetation in an area (Harp and Petranka, 2006; Gray et al., 2007; Greer and Collins, 2008; Miller et al., 2011). For a state like Oklahoma, where pasture and rangeland make up approximately 50% of the land use in the state, oil and natural gas extraction are prevalent, and wildlife-related recreational activities are common (U.S. Department of the Interior, 2001; U.S. Department of Agriculture, 2017; U.S. Energy Information Administration, 2020), these anthropogenic factors are likely contributing collectively to the distribution and prevalence of both *Bd* and RV.

Amphibians in Oklahoma have been exposed to *Bd* for several decades (Watters et al., 2016), although the extent of disease prevalence across the state has become more well understood only recently through regional and statewide surveys (Marhanka et al., 2017; Davis et al., 2018; Watters et al., 2018, 2019; Smith et al., 2019). However, no study to date has assessed patterns of disease prevalence and distribution at a statewide level, nor has the impact of human-mediated habitat disturbance on pathogen threats of amphibian populations been evaluated. Oklahoma is home to many national wildlife refuges, state-managed wildlife management areas and state parks, and other conservation lands, all exposed to varying levels of anthropogenic impacts that may be contributing to amphibian disease spread and regionalized population susceptibility. In this study, we assess whether disease prevalence and pathogen load across Oklahoma has a positive correlation with the degree of human-mediated environmental impact. Such a correlation would indicate that amphibian populations in environments increasingly impacted by human-mediated stressors will be at a greater danger for mass mortality events and regional extirpation in the future (Leung et al., 2017).

Methods

Field Data Collection

Fieldwork was conducted in March–June and September–October in 2015, March–June in 2016, and March–June and October in 2017. Surveys were conducted around ponds, lakes, streams, and wetlands in Oklahoma Department of Wildlife (ODWC) Wildlife Management Areas (WMAs), National Wildlife Refuges (NWR), Oklahoma State Parks (SP), and The Nature Conservancy (TNC) preserves (Figure 1). This study is a meta-analysis and field study, combining reported data for central, northeastern, and southeastern Oklahoma (Marhanka et al., 2017; Davis et al., 2018;

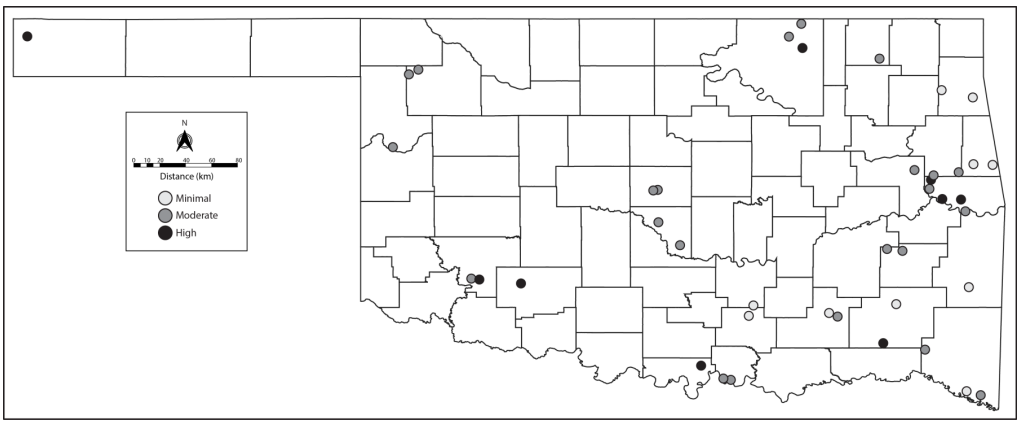

Figure 1. Map of Oklahoma, USA showing sampled sites for the study, with different colored circles representing minimal (light gray), moderate (dark gray), or high (black) human impact for each site. If multiple waterbodies were sampled within a site, the placement of each circle for a sampled area corresponds to the most sampled pond or lake at that site.

Watters et al., 2018, 2019; Smith et al., 2019), along with unpublished disease survey data for amphibian populations in western Oklahoma and TNC preserves. Overall, 43 distinct sites were sampled (Figure 1), with 3–179 individuals sampled per site (total N = 1,514 individuals for *Bd*; total N = 1,526 individuals for RV).

Sites were classified as having minimal, moderate, or high human impact based on the type and volume of human activity in that area (Table 2). Minimal human impact environments were defined as sites often forbidding public access, though some locations may allow minimal hunting or fishing (N = 11 sites). Moderate human impact environments were defined as sites having some amount of grazing, recreation, boating, fishing, hunting, and oil and/or natural gas exploitation, with waterbodies located near roadways and recreational parks (N = 23 sites). The volume of traffic at these sites was higher than at sites of minimal impact, but recreational use was not heavy on a frequent basis. High human impact environments were defined as sites subject to heavy mining and oil and/or natural gas extraction, grazing, fishing, recreational vehicle usage, hunting, and/or boating (N = 9 sites). All Oklahoma State Parks were designated as high human impact sites. Human impact status decisions were based on communications with land managers, public website descriptions of sampled locations, and our direct field observations (ODWC, 2020). There were no available statistics for numbers of visitors for most sites included in this study.

Each site was surveyed for a total of 12–48 h, with amphibians captured by hand, aquatic trap, dip net, or seine, and then kept in individual plastic bags until swabbing and euthanization or release. All field collecting equipment (i.e. waders, traps, nets) was sterilized between locations using 10% bleach to avoid any potential contamination of sites (Gray et al., 2017). In most cases, amphibians were transported back to the Sam Noble Oklahoma Museum of Natural History (SNOMNH) prior to disease sample collection. Rayon-tipped swabs (Medical Wire, MWE 113) were rubbed over the surface of the live amphibians before euthanization, for the collection of potential *Bd* spores (Lannoo et al., 2011). Swab heads were placed into sterile, individually labeled 1.5mL vials. In most cases, animals were then immediately euthanized via an aqueous solution of chloretone (hydrous chlorobutanol; 1, 1, 1, Tri-Chlora 2-methyl, 2-propanol), containing 1 teaspoon of crystals per 500mL of DI water, for a length of time appropriate to their body size and skin thickness, usually 3–5 minutes (Simmons, 2015). For those amphibians caught as part of a repeat sampling project in central Oklahoma, many individuals were released on-site after swabbing (Smith et al., 2019; Watters et al., 2019). In order to screen for ranavirus, a tissue sample was collected, either from the liver of euthanized animals or the tail or toe of released animals (St-Amour and Lesbarreres, 2007). Tissue samples were flash frozen in liquid nitrogen or preserved in 95% ethanol, then stored in 2mL cryovials. DNA from swabs were extracted using PrepMan Ultra (Applied Biosystems; Cheng et al., 2011) and tissue samples were extracted using a high salt extraction method (Esselstyn et al., 2008). All reusable equipment (i.e. scissors, forceps) was sterilized between sample collections using 10%

Table 1. Nucleotide sequence for forward primers, reverse primers, and probes used in *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus (RV) screening in this study (Boyle et al., 2004; Forson and Storfer, 2006).

	Bd	**RV**
Forward primer	CCTTGATATAATACAGTGTGCCARARGTC	ACACCACCGCCCAAAAGTAC
Reverse Primer	AGCCAAGAGATCCGTTGTCAAA	CCGTTCATGATGCGGATAATG
Probe	CGAGTCGAACAAAAT	CCTCATCGTTCTGGCCATCAACCAC

bleach or Eliminase (DeconLabs); gloves were also changed between each sample collection (Gray et al., 2017).

Genetic Analysis

Quantitative PCR (qPCR) methodologies were employed to determine presence of *Bd* and RV genetic signatures from swab or tissue extractions and to estimate the number of gene copies per sample (infection load) using protocols from Kerby et al. (2013). DNA extracts were diluted 1:10 for *Bd* and 1:1 for RV with 0.25x TE Buffer to remove potential inhibitors. Samples from 2015 were analyzed at the Disease Testing and Sequencing Facility at the University of South Dakota (StepOnePlus Real-Time PCR, software v2.3); 2016–2017 samples were analyzed at the SNOMNH Genomics Core Facility (QuantStudio 3.0 Design and Analysis Software). For each qPCR run, samples for *Bd* and RV were run in triplicate, along with positive controls containing known gene copy numbers for both pathogens (gBlock DNA quantities $1e^1$–$1e^4$), and a single negative control (ddH_2O). For *Bd*, primers targeted the ITS-1 rRNA gene (Boyle et al., 2004), and for RV, primers targeted the major capsid protein (MCP; Forson and Storfer, 2006) (Table 1). Samples were considered positive for *Bd* (*Bd*+) or RV (RV+) if amplification occurred in at least two of the three wells and if the mean gene copy number per well (from qPCR output) was greater than 1.0. Any samples that tested positive in only one of three qPCR wells was re-run on a new qPCR plate, to determine whether it was a true negative or whether the pathogen DNA was simply present in very small quantities; if the rerun resulted in at least one positive well, the sample was considered positive. Infection load was calculated by obtaining the mean gene number copy/sample from all wells indicating positive results (from the qPCR analysis software), then multiplying this value by the original extract volume, and the appropriate dilution factor. Disease prevalence data was analyzed by human impact level using non-parametric Kruskal-Wallis tests for each pathogen. Additional Kruskal-Wallis tests were performed on infection load (mean gene copies/sample) by human impact level, with pairwise Wilcoxon-Rank Sum analyses performed as needed.

Results

The results of our metanalysis show an average pathogen prevalence of 47% and 19.2% for *Bd* and RV, respectively, across the state of Oklahoma (Table 2). Prevalence patterns for *Bd* in Oklahoma were not correlated significantly with environmental human impact level, despite a visible stair-step trend of increased prevalence when moving from minimal to high human impact ($H = 1.65$, $P = 0.438$; Figure 2). Although RV prevalence data also showed an increasing trend from minimal to moderate levels, high human impact environments possessed similar viral prevalence to moderate human impact sites (Figure 2); however, these trends were not statistically significant ($H = 2.86$, $P = 0.239$). With regard to infection load, there was no observed significant correlation to environmental human impact level for *Bd* ($H = 5.63$, $P = 0.0702$; Figure 3), although sites of moderate human impact tended to have individuals with higher infection loads (Table 2). In contrast, we do observe a statistically significant correlation between RV infection load and human impact level ($H = 28.9$, $P < 0.001$; Table 2; Figure 3), with post-hoc comparisons of RV infection load supporting a significant difference between sites of minimal and moderate human impact only ($z = 2.21$, $P = 0.0135$).

Discussion

Sampling efforts aimed at monitoring *Bd* and RV in Oklahoma have shown that the two diseases affect herpetofauna in communities across the state, with *Bd* found at higher prevalence levels than RV (Marhanka et al., 2017; Davis et al., 2018; Watters et al., 2018, 2019; Smith et al., 2019). Our meta-analysis found a non-significant trend of increasing prevalence in both *Bd* and RV as the degree of human impact on surveyed environments increased (Figure 2). Although we could not fully reject our null hypothesis, additional surveys across a greater temporal sampling of communities for disease may show more significant associations with

direct and indirect human-mediated disturbances (Brittingham et al., 2014; Gray et al., 2017). Still, the observed trend of increased prevalence with increased human impact remains relevant to local land managers. Direct spread by human recreational activities could be mitigated through increased education regarding disinfection for people visiting the various locations and/or purchasing hunting and fishing permits for use in the state (Casais et al., 2019; Bienentreau and

Table 2. List of all Oklahoma sites sampled for *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus (RV) prevalence from 2015–2017, sorted by human impact level. Total sample size, number of positive individuals (+), prevalence (%) ± standard deviation (SD), and mean ($\bar{X}$) infection load ± SD of infection load are listed for *Bd* and RV for each site. Site abbreviations are as follows: Oklahoma Department of Wildlife (ODWC) Wildlife Management Areas (WMAs), National Wildlife Refuges (NWR), Oklahoma State Parks (SP), and The Nature Conservancy (TNC) preserves.

		Bd				RV			
Site name	**County**	**N**	**+**	**%**	**$\bar{X}$ infection load (± SD)**	**N**	**+**	**%**	**$\bar{X}$ infection load (± SD)**
MINIMAL		545	166	30.46	659,181.95 (± 2,897,358.30)	546	46	8.42	37,137.53 (± 143,442.36)
Grassy Slough WMA (33.78324, -94.76353)	McCurtain	27	20	74.07	275,147.29 (± 449,154.80)	27	2	7.41	301.69 (± 267.07)
Oka'yanahli TNC (34.43442, -96.64560)	Johnston	177	23	12.99	10,095.09 (± 32,364.02)	179	0	0	N/A (± N/A)
Ouachita WMA (34.68206, -94.74407)	Le Flore	54	25	46.3	899,866.83 (± 2,086,748.90)	14	1	7.14	586.10 (± 692.33)
Ozark Plateau NWR, Hamby Unit (36.31092, -94.70803)	Delaware	40	14	35	7,778.27 (± 136,431.57)	38	4	10.53	337,939.87 (± 150,252.20)
Ozark Plateau NWR, vicinity of Night Train Farm (35.74396, -94.70409)	Delaware	14	7	50	278,477.52 (± 632,016.70)	13	1	7.69	10,062.53 (± N/A)
Ozark Plateau NWR, Looney Unit (36.32050, -94.70953)	Adair	47	23	48.94	43,902.14 (± 84,784.26)	47	0	0	N/A (± N/A)
Ozark Plateau NWR, Sallybull Unit (35.73804, -94.53851)	Adair	20	6	30	1,897.25 (± 2,473.99)	19	0	0	N/A (± N/A)
Pontotoc Ridge TNC (34.52409, -96.60590)	Pontotoc	38	2	5.26	2,577.39 (± 3,361.56)	39	4	10.26	1,952.54 (± 2,164.88)
Pushmataha WMA (34.53523, -95.37177)	Pushmataha	47	24	51.06	2,505,419.96 (± 6,128,539.50)	49	14	28.57	1,305.41 (± 1,788.50)
Spavinaw WMA (36.38328, -94.97942)	Delaware	69	17	24.64	28,310.38 (± 65,379.88)	70	3	4.29	3,109.07 (± 1,459.82)
Stringtown WMA (34.45893, -95.95204)	Atoka	12	5	41.67	505,698.71 (± 815,319.18)	12	5	7.41	413.28 (± 367.44)
MODERATE		788	417	52.92	1,013,172.80 (± 7,347,673.10)	821	207	25.21	523,690.96 (± 4,657,370.82)
50th St. & Bartell Ave., Oklahoma City (35.52229, -97.43267)	Oklahoma	37	23	62.16	5,568,914.14 (± 23,237,913)	34	4	11.76	74,749.52 (± 147,447.02)
Arkansas River at Robert S. Kerr Lock and Dam 15 (35.52229, -97.43267)	Le Flore	14	9	64.29	843,889.22 (± 1,599,005.30)	14	1	7.14	202.96 (± N/A)
Camp Gruber WMA (35.69351, -95.21388)	Muskogee	28	21	75	165,010.91 (± 555,831.77)	28	21	75	38,619.80 (± N/A)
Cherokee WMA (35.64775, -95.04848)	Cherokee	14	1	7.14	76.64 (± N/A)	14	0	0	N/A (± N/A)
Cookson WMA (35.67467, -94.83175)	Adair/ Cherokee	142	89	62.68	N/A (± N/A)	151	54	35.76	N/A (± N/A)
Cooper WMA (36.56062, -99.50166)	Woodward	10	1	10	1,984.38 (± 995.25)	10	0	0	N/A (± N/A)

Table 2. Continued

Fobb Bottom WMA (33.89288, -96.86565)	Marshall	3	1	33.33	277,499.40 (± N/A)	3	1	33.3	496.68 (± N/A)
Fort Supply WMA (36.51990, -99.58335)	Woodward	4	1	25	1,968.00 (± N/A)	4	1	25	8,649.65 (± N/A)
Hulah WMA (36.95639, -96.19199)	Osage	11	4	36.36	N/A (± N/A)	11	4	36.36	N/A (± N/A)
James Collins WMA (35.01187, -95.45086)	Latimer	26	14	53.85	93,967.58 (± 162,633.49)	33	6	18.18	666.18 (± 370.12)
Lexington WMA (35.04437, -97.24004)	Cleveland	47	18	39.3	19,439.89 (± 27,500.96)	46	9	19.57	847.76 (± 1,219.16)
McClellan-Kerr WMA (35.53111, -95.08433)	Sequoyah	30	8	26.67	47,133.66 (± 73,122.65)	30	8	26.67	7,432.80 (± 6,688.91)
McGee Creek WMA (34.42796, -95.87825)	Atoka	29	19	65.52	255,365.08 (± 456,852.75)	30	4	13.33	459.22 (± 318.31)
Mountain Park WMA (34.75610, -99.04420)	Kiowa	18	1	5.56	24,856.20 (± 35,002.75)	18	0	0	N/A (± N/A)
Oklahoma City Zoo (35.51705, -97.47129)	Oklahoma	59	34	57.63	1,477,820.27 (± 6,219,816.90)	60	17	28.33	337.68 (± 455.31)
Oologah WMA (36.65560, -95.51593)	Nowata	62	26	41.94	191,331.69 (± 643,974.46)	62	26	41.94	5,588.7 (± N/A)
Osage Hills WMA (36.74756, -96.18187)	Osage	14	14	100	337,368.63 (± 558,724.25)	20	0	0	N/A (± N/A)
Packsaddle WMA (35.89135, -99.72193)	Ellis	14	13	92.86	283,679.73 (± 706,615.74)	14	2	14.29	245.35 (± 159.75)
Pine Creek WMA (34.14252, -95.12228)	McCurtain	16	5	31.25	46,234.47 (± 65,356.31)	21	16	24.53	2,455,477.09 (± 10,979,101.38)
Red Slough WMA (33.74901, -94.64159)	McCurtain	58	48	82.76	2,302,191.52 (± 8,603,747.50)	2	2	76.19	413.70 (± 363.21)
Robbers Cave WMA (34.99551, -95.31754)	Latimer	22	13	59.1	169,048.50 (± 245,605.34)	29	9	31.03	1,305,693.70 (± 3,904,431.08)
Sutton Urban Wilderness, (35.24266, -97.42689)	Cleveland	103	28	27.18	122,211.16 (± 326,647.18)	105	15	14.29	481.62 (± 558.68)
University of Oklahoma Biological Station (33.88150, -96.80122)	Marshall	27	26	96.3	5,890.84 (± 12,564.51)	26	0	0	N/A (± N/A)
HIGH		181	129	71.27	268,968.75 (± 955,328.46)	159	41	25.79	40,249.25 (± 102,739.85)
Black Mesa SP (36.84771, -102.88154)	Cimarron	9	4	44.44	91,993.60 (± 106,275.25)	10	5	50	7,523.29 (± 2,164.88)
Great Plains SP (34.74799, -98.97459)	Kiowa	5	0	0	N/A (± N/A)	5	1	20	2,507.95 (± N/A)
Hickory Creek WMA (34.00422, -97.05785)	Love	35	35	100	11,508.19 (± 41,099.02)	33	2	6.06	180.80 (± 88.63)
Hugo WMA (34.19843, -95.48391)	Choctaw/ Pushmataha	3	1	33.33	598.24 (± N/A)	2	2	100	459.89 (± 443.54)
KOA Group Campground, Sallisaw (35.43885, -94.81166)	Sequoyah	6	4	66.67	N/A (± N/A)	6	0	0	N/A (± N/A)
Osage Hills SP (36.74756, -96.18187)	Osage	35	26	74.29	24,048.69 (± N/A)	20	12	60	N/A (± N/A)
Sequoyah NWR (35.44331, -94.97335)	Sequoyah	28	10	35.71	10,837.95 (± 2,688.18)	28	12	42.86	93,658.03 (± 150,252.20)
Tenkiller WMA & SP (35.60904, -95.07096)	Sequoyah	4	1	25	281,947.92 (± N/A)	4	0	0	N/A (± N/A)
Wichita Mountains NWR (34.71400, -98.61472)	Comanche	56	48	85.71	524,981.54 (± 1,321,403.6)	51	7	13.73	5,271.92 (± 7,834.56)
TOTAL		**1,514**	**712**	**47.03**	**782,940.66 (± 5,615,695.70)**	**1,526**	**294**	**19.27**	**331,055.59 (± 3,619,434.80)**

Lesbarreres, 2020).

Interestingly, infection load data for both diseases was highest at moderate human impact sites (Figure 3). This effect was more pronounced for RV data, with significant differences between RV gene copies when comparing minimal and moderate environmental disturbances (Figure 3). Studies have proposed that stress to amphibian immune systems from human traffic, grazing, and land use can lead to infection load data mirroring disease prevalence data as human interaction with the environment increases (Brittingham et al., 2014; Gray et al., 2017; Bienentreau and Lesbarreres, 2020). One possible explanation for our observed results is that many individuals with higher infection loads may have already succumbed to the disease but not have been observed in mortality events. More research needs to be done to further elucidate the relationship between infection load and pathogen prevalence for both *Bd* and RV, and the interplay with direct and indirect human impacts (Warne et al., 2016; Bienentreau and Lesbarreres, 2020). Unfortunately, host responses to disease infection involve a myriad of environmental and host-specific factors, all of which together result in high variability of disease outcomes observed in field settings, making predictive assumptions difficult (Zamudio et al., 2020).

Future research to assess anthropogenic impacts on *Bd* and RV prevalence and infection load could be improved with improved assessment and temporal monitoring of human impacts on environmental health across the state, as well as more even sampling of amphibian communities and sites within disturbance categories. For example, Robbers Cave WMA exhibited a higher-than-average prevalence for both pathogens (Table 2), and human impacts on-site include natural gas pipelines, hunting, and fishing (Watters, personal observation; ODWC, 2020). However, our study did not address the impacts of each occurrence individually, merely as a whole. Assessing water quality, quantifying environmental contaminants, measuring distance from a pond to a pipeline, or counting numbers of fisherman and hunters per year would improve our understanding of individual disturbance activity impacts. Additionally, should more detailed information about daily traffic and levels of recreational use across the state become available, future studies may be able to re-evaluate the large, moderate impact category with more precision. Unfortunately, at present, the vast majority of sites have no metric by which to measure human visitation as they are broadly open to the public (no locked gates, etc.). However, as of June 2020, Oklahoma State Parks require a daily parking pass, which may at least provide relative data for comparison across parks.

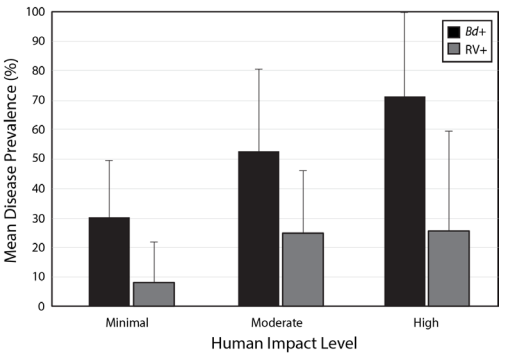

Figure 2. Comparisons of *Batrachochytrium dendrobatidis* (*Bd*+) and ranavirus (RV+) mean prevalence (%) with standard error bars among sites of minimal, moderate, and high human impact.

Although the observed trends along the human impact gradient lacked statistical significance, this could be the result of smaller sample sizes per site or category. For example, some sites in the moderate and high impact categories had fewer than 10 individual disease samples collected (Table 2). Additionally, there were comparatively fewer locations of minimal and high human impact (N = 11 and 9, respectively) compared to the number of locations classified as having moderate human impact (N = 23). The addition of increased species-specific and community-level sampling across sites, as well as additional surveys at minimal and high human impact environments, would allow for more robust tests for correlated patterns. In general, more objective classifications of human impact based on environmental measurements

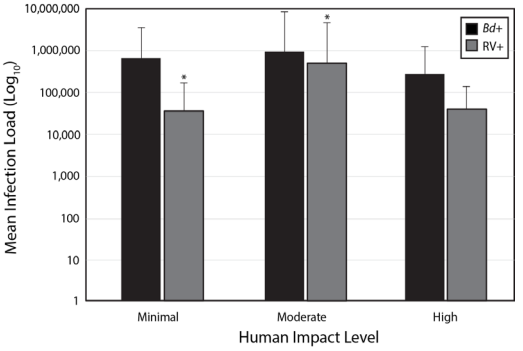

Figure 3. Comparisons of *Batrachochytrium dendrobatidis* (*Bd*+) and ranavirus (RV+) mean infection loads (calculated from mean gene copies) with standard error bars among sites of minimal, moderate, and high human impact. Statistical analysis resulted in a significant difference of RV infection loads between sites of minimal and moderate human impact only, as indicated by an asterisk (*).

and recreational and land usage metrics would increase statistical power in future studies. With identification of the most influential behaviors on human-mediated spread of *Bd* and RV, we can direct education efforts to land managers and the public accordingly.

In conclusion, our findings will help conservation efforts within Oklahoma by identifying specific areas in need of further preventive measures against the spread of amphibian infectious disease. Disease prevalence at several moderate and high areas of human impact are above 50% for *Bd* and around 25% for RV (Table 2). Additionally, since RV is not specific to amphibians, it has the potential to infect sympatric reptiles (particularly turtles) and fish in their ecosystems (Jancovich et al., 2005; Currylow et al., 2014), therefore, continued monitoring is recommended for all taxa potentially impacted. Additionally, while preliminary results on amphibian infectious diseases have been shared through an ODWC blog in 2018 (https://www.wildlifedepartment.com/ooj/health-checkup-oklahomas-frogs-and-salamanders), an official educational campaign should be developed for Oklahoma. This could include instructions for fisherman or boaters on properly sterilizing equipment when moving between bodies of water (Cunningham et al., 2003; Gray et al., 2017) and on the threats of using amphibians as bait that may carry infections as has been documented in other areas of the United States (Jancovich et al., 2005; Picco and Collins, 2008). Research indicates that the only way to mitigate amphibian population declines is to improve communication and collaboration among all possible stakeholders—researchers, land owners and managers, and the general public alike (Canessa et al., 2019).

Acknowledgments

We thank Siler Lab members for assistance in field sampling, DNA extractions, and qPCR screening necessary for completion of this project. We also thank the University of Oklahoma Honors College and the Sam Noble Oklahoma Museum of Natural History for providing SEH the opportunity to work on this project with the Siler Lab. Specimens were collected under applicable Oklahoma Department of Wildlife Scientific Collecting Permits to JLW and CDS, and the methodologies were approved by the University of Oklahoma IACUC Committee (#R14-026). Funding was provided to CDS and JLW by the Oklahoma Department of Wildlife Conservation (grant number F14F01225 [T-80-1]) and an Oklahoma City Zoo and Botanical Gardens Conservation Action Now grant. Lastly, we would like to thank two anonymous reviewers for their edits on this manuscript.

References

Beebee TJC, Griffiths RA. 2005. The amphibian decline crisis: a watershed for conservation biology? Biol Conserv 125: 271–285. https://doi.org/10.1016/j.biocon.2005.04.009

Bienentreau J-F, Lesbarrères D. 2020. Amphibian disease ecology? Are we just scratching the surface? Herpetologica 76(2): 153–166. https://doi.org/10.1655/0018-0831-76.2.153

Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, et al., 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the train forests of Australia and Central America. PNAS 95: 9031–9036. https://doi.org/10.1073/pnas.95.15.9031

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Organ 60: 141–148. https://doi.org/10.3354/dao060141

Brittingham MC, Maloney KO, Farag AM, Harper DD, Bowen ZH. 2014. Ecological risks of shale oil and gas development to wildlife, aquatic resources and their habitats. Environ Sci 48: 11034–11047. https://doi.org/10.1021/es5020482

Grant EHC, Miller DAW, Muths E. 2020. A synthesis of evidence of drivers of amphibian decline. Herpetologica 76(2): 101–107. https://doi.org/10.1655/0018-0831-76.2.101

Canessa S, Spitzen-van der Sluijs A, Martel A, Pasmans F. 2019. Mitigation od amphibian disease requires a stronger connection between research and management. Biol Conserv 236: 236–242. https://doi.org/10.1016/j.biocon.2019.05.030

Casais R, Larrinaga AR, Dalton KP, Domínguez Lapido R, Márquez I, Bécares E, Carter ED, Gray MJ, Miller DL, Balseiro A. 2019. Water sports could contribute to the translocation of ranaviruses. Sci Rep-UK 9: 2340. https://doi.org/10.1038/s41598-019-39674-5

Cheng TL, Rovito SM, Wake DB, Vredenburg VT. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. PNAS 108: 9502–9507. https://doi.org/10.1073/pnas.1105538108

Cunningham AA, Daszak P, Rodriguez JP. 2003. Pathogen pollution: defining a parasitological threat to biodiversity conservation. J Parasitol 89: 878–883. https://www.researchgate.net/publication/284061023

Currylow AF, Johnson AJ, Williams RN. 2014. Evidence of ranavirus infections among sympatric larval amphibians and box turtles. J Herpetol 48: 117–121. http://dx.doi.org/10.1670/12-235

Davis DR, Farkas JK, Kruisselbrink TR, Watters JL, Ellsworth ED, Kerby JL, Siler CD. 2018. Prevalence and distribution of ranavirus in amphibians from southeastern Oklahoma, USA. Herpetol Conserv Biol 14: 360–369. www.herpconbio.org/Volume_14/Issue_2/Davis_etal_2019.pdf

Esselstyn JA, Garcia HJD, Saulog MG, Heaney LR. 2008. A new species of *Desmalopex* (Pteropodidae) from the Philippines, with a phylogenetic analysis of the Pteropodini. J Mammal 89: 815–825. https://doi.org/10.1644/07-MAMM-A-285.1

Fisher MC, Garner TWJ. 2007. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. Fungal Biol Rev 21: 2–9. . https://doi.org/10.1016/j.fbr.2007.02.002

Ford J, DAGA Hunt, Haines GE, Lewis M, Lewis Y, Green DM. 2020. Adrift on a sea of troubles: can amphibians survive in a human-dominated world? Herpetologica 76(2): 251–256. https://doi.org/10.1655/0018-0831-76.2.251

Forson DD, Storfer A. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum.* Ecol Appl 16: 2325–2332. https://doi.org/10.1890/1051-0761(2006)016[2325:AIRSIT]2.0.CO;2

Grant EHC, Miller DAW, Muths E. 2020. A synthesis of evidence of drivers of amphibian decline. Herpetologica 76(2): 101–107. https://doi.org/10.1655/0018-0831-76.2.101

Gray, MJ, Chinchar VG. 2015. Ranaviruses: lethal pathogens of ectothermic vertebrates. New York (NY): Springer International Publishing. 256 p.

Gray MJ, Miller DL, Schmutzer AC, Baldwin CA. 2007. *Frog virus 3* prevalence in tadpole populations inhabiting cattle-access and non-access wetlands in Tennessee, USA. Dis Aquat Organ 77: 97–103. https://doi.org/10.3354/dao01837

Gray MJ, Miller DL, Hoverman JT. 2009. First report of ranavirus infecting lungless salamanders. Herpetol Rev 40: 316–319.

Gray MJ, Duffus ALJ, Haman KH, Harris RN, Allender MC, Thompson TA, Christman MR, Sacerdote-Velat A, Sprague LA, Williams JM, Miller DL. 2017. Pathogen surveillance in herpetofaunal populations: guidance on study design, sample collection, biosecurity, and intervention strategies. Herpetol Rev 48: 334–351.

Green DM, Lannoo MJ, Lesbarrères D, Muths E. 2020. Amphibian population declines: 30 years of progress in confronting a complex problem. Herpetologica 76(2): 97–100. https://doi.org/10.1655/0018-0831-76.2.97

Greer AL, Collins JP. 2008. Habitat fragmentation as a result of biotic and abiotic factors controls pathogen transmission throughout a host population. J Anim Ecol 77: 364–369. https://doi.org/10.1111/j.1365-2656.2007.01330.x

Guo F, Bonebrake TC, Gibson L. 2018. Land-use change alters host and vector communities and may elevate disease risk. EcoHealth 16: 647–658. https://doi.org/10.1007/s10393-018-1336-3

Han BA, Searle CL, Blaustein AR. 2011. Effects of an infectious fungus, *Batrachochytrium dendrobatidis*, on amphibian predator-prey interactions. PLOS One 6(2): e16675. https://doi.org/10.1371/journal.pone.0016675

Harp EM, Petranka JW. 2006. Ranavirus in wood frogs (*Rana sylvatica*): potential sources of transmission within and between ponds. J Wildl Dis 42(2): 307–318. https://doi.org/10.7589/0090-3558-42.2.307

Hoverman JT, Gray MJ, Haislip NA, Miller DL. 2011. Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth 8: 301–319. https://doi.org/10.1007/s10393-011-0717-7

Jancovich JK, Davidson EW, Parameswaran N, Mao J, Chinchar VG, Collins JP, Jacobs BL, 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

Kerby JL, Storfer A. 2009. Combined effects of atrazine and chlorpyrifos on susceptibility of the tiger salamander to *Ambystoma tigrinum* virus. EcoHealth 6: 91–98. https://doi.org/10.1007/s10393-009-0234-0

Kerby JL, Hart AJ, Storfer A. 2011. Combined effects of virus, pesticide, and predator cue on the larval tiger salamander (*Ambystoma tigrinum*). EcoHealth 8: 46–54. https://doi.org/10.1007/s10393-011-0682-1

Kerby JL, Schieffer A, Brown JR, Whitfield S. 2013. Utilization of fast qPCR techniques to detect the amphibian chytrid fungus: a cheaper and more efficient alternative method. Methods Ecol Evol 4: 162–166. https://doi.org/10.1111/j.2041-210x.2012.00263.x

Lannoo MJ, Petersen C, Lovich RE, Nanjappa P, Phillips C, Mitchell JC, Macallister I. 2011. Do frogs get their kicks on route 66? Continental U.S. transect reveals spatial and temporal patterns of *Batrachochytrium dendrobatidis* infection. PLOS One 6: e22211. https://doi.org/10.1371/journal.pone.0022211

Leung WTM, Thomas-Walters L, Garner TWJ, Balloux F, Durrant C, Price SJ. 2017. A quantitative-PCR based method to estimate ranavirus viral load following normalization by reference to an ultraconserved vertebrate target. J Virol Methods 249: 147–155. https://doi.org/10.1016/j.jviromet.2017.08.016

Marhanka EC, Watters JL, Huron NA, McMillin SL, Winfrey CC, Curtis DJ, Davis DR, Farkas JK, Kerby JL, Siler CD. 2017. Detection of high prevalence of *Batrachochytrium dendrobatidis* in amphibians from southern Oklahoma, USA. Herpetol Rev 48: 70–74.

McMenamin SK, Hadly EA, Wright CK. 2008. Climatic change and wetland desiccation cause amphibian decline in Yellowstone National Park. PNAS 105(44): 16988–16993. https://doi.org/10.1073/pnas.0809090105

Miller D, Gray M, Storfer A. 2011. Ecopathology of ranaviruses infecting amphibians. Viruses 3(11): 2351–2373. https://doi.org/10.3390/v3112351

O'Hanlon SJ, Rieux A, Farrer RA, Rosa GM, Waldman B, Bataille A, Kosch TA, Murray KA, Brankovics B, Fumagalli M, et al., 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. Science 360: 621–627. https://doi.org/10.1126/science.aar1965

Oklahoma Department of Wildlife Conservation (ODWC). 2020. Wildlife Management Areas [online]. Available from https://www.wildlifedepartment.com/hunting/wma (Accessed December 14, 2020).

Picco A, Collins JP. 2008. Amphibian commerce as a likely source of pathogen pollution. Conserv Biol 22: 1582–1589. https://doi.org/10.1111/j.1523-1739.2008.01025.x

Pounds JA, Bustamante MR, Coloma LA, Consuegra JA, Fogden MPL, Foster PN, La Marca E, Masters KL, Merino-Viteri A, Puschendorf R, et al., 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439: 161–167. https://doi.org/10.1038/nature04246

Price SJ, Garner TWJ, Cunningham AA, Langton TES, Nichols RA. 2016. Reconstructing the emergence of a lethal infectious disease of wildlife supports a key role for spread through translocations by humans. Proc R Soc B 283: 20160952. https://doi.org/10.1098/rspb.2016.0952

Robert J, McGuire CC, Nagel S, Lawrence BP, De Jesús Andino F. 2019. Developmental exposure to chemicals associated with unconventional oil and gas extraction alters immune homeostasis and viral immunity of the amphibian *Xenopus*. Sci Total Environ 671: 644–654. https://doi.org/10.1016/j.scitotenv.2019.03.395

Simmons JE. 2015. Herpetological Collecting and Collections Management. Third Edition. Salt Lake City (UT): Society for the Study of Amphibians and Reptiles (SSAR). 191 p.

Schloegel LM, Picco AM, Kilpatrick AM, Davies AJ, Hyatt AD, 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

Smith SN, Watters JL, Marhanka EC, McMillin SL, Davis DR, Farkas JK, Siler CD. 2019. Investigating seasonal variation of ranavirus in central Oklahoma, USA, amphibians. Herpetol Rev 50(3): 508–512.

Souza MJ, Gray MJ, Colclough P, Miller DL. 2012. Prevalence of infection by *Batrachochytrium dendrobatidis* and ranavirus in eastern hellbenders (*Cryptobranchus alleganiensis*) in eastern Tennessee. J Wildl Dis 48: 560–566. https://doi.org/10.7589/0090-3558-48.3.560

St-Amour V, Lesbarrères D. 2007. Genetic evidence of ranavirus in toe clips: an alternative to lethal sampling methods. Conserv Genet 8: 1247–1250. https://doi.org/10.1007/s10592-006-9242-6

U.S. Department of Agriculture. 2017. 2017 Natural Resources Inventory. Natural Resources Conservation Service, Washington, DC [online]. Available from https://www.nrcs.usda.gov/Internet/NRCS_RCA/reports/nri_ok.html (Accessed November 9, 2020).

U.S. Fish and Wildlife Service. 2011. U.S. Department of the Interior, U.S. Fish and Wildlife Service, and U.S. Department of Commerce, U.S. Census Bureau: 2011 National Survey of Fishing, Hunting, and Wildlife-Associated Recreation: Oklahoma [online]. Available from https://digitalmedia.fws.gov/digital/collection/document/id/1695/ (Accessed December 14, 2020).

U.S. Energy Information Administration. 2020. Oklahoma State Energy Profile [online]. Available from https://www.eia.gov/state/print.php?sid=OK (Accessed November 9, 2020).

Venesky MD, Wassersug RJ, Parris MJ. 2010. Fungal pathogen changes the feeding kinematics of larval anurans. J Parisitol 96(3): 552–557. https://doi.org/10.1645/GE-2353.1

Voyles J, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, Cook D, Webb R, Alford RA, Skerratt LF, et al. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326: 582–585. https://doi.org/10.1126/science.1176765

Warne RW, Crespi EJ, Brunner JL. 2011. Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. Funct Ecol 25: 139–146. https://doi.org/10.1111/j.1365-2435.2010.01793.x

Warne RW, LaBumbard B, LaGrange S, Vredenburg VT, Catenazzi A. 2016. Co-infection by chytrid fungus and *ranaviruses* in wild and harvested frogs in the tropical Andes. PLOS One 11: e0145864. https://doi.org/10.1371/journal.pone.0145864

Watters JL, Flanagan RL, Davis DR, Farkas JK, Kerby JL, Labonte MJ, Penrod ML, Siler CD. 2016. Screening natural history collections for historical presence of *Batrachochytrium dendrobatidis* in anurans from Oklahoma, USA. Herpetol Rev 47: 214–220.

Watters JL, Davis DR, Yuri T, Siler CD. 2018. Concurrent infection of *Batrachochytrium dendrobatidis* and ranavirus among native amphibians from northeastern Oklahoma, USA. J Aquat Anim Health 30: 291–301. https://doi.org/10.1002/aah.10041

Watters JL, McMillin SL, Marhanka EC, Davis DR, Farkas JK, Kerby JL, Siler CD. 2019. Seasonality in *Batrachochytrium dendrobatidis* detection in amphibians in central Oklahoma, USA. J Zoo Wildlife Med 50: 492–497. https://doi.org/10.1638/2018-0103

Weldon C, du Preez LH, Hyatt AD, Muller R, Speare R. 2004. Origin of the amphibian chytrid fungus. Emerg Infect Dis 10: 2100–2105. https://doi.org/10.3201/eid1012.030804

Whitfield SM, Geerdes E, Chacon I, Rodriguez EB, Jimenez RR, Donnelly MA, Kerby JL. 2013. Infection and co-infection by the amphibian chytrid fungus and ranavirus in wild Costa Rican frogs. Dis Aquat Organ 104: 173–178. https://doi.org/10.3354/dao02598

Zamudio KR, McDonald CA, Belasen AM. 2020. High variability in infection mechanisms and host responses: a review of functional genomic studies of amphibian chytridiomycosis. Herpetologica 76(2): 189–200. https://doi.org/10.1655/0018-0831-76.2.18.

Submitted December 16, 2020 Accepted July 29, 2021