2016

Aeromonas hydrophila In Amphibians: Harmless Bystander or Opportunistic Pathogen

Zachary P. Rivas
University of Central Florida

AEROMONAS HYDROPHILA IN AMPHIBIANS: HARMLESS BYSTANDER OR OPPURTUNISTIC PATHOGEN?

by

ZACHARY P. RIVAS

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Biology
in the College of Arts and Sciences
and in The Burnett Honors College
at the University of Central Florida
Orlando, Florida

Spring Term, 2016

Thesis Chair: Dr. Anna E. Savage

ABSTRACT

For several decades amphibian populations have been declining. Historically, the bacterium *A. hydrophila* (*Ah*) was hypothesized to be the causal factor in amphibian disease and population declines. However, with the discovery of a chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) in 1998, which was identified on the skin of amphibians during documented mortality events, *Ah* research became of minor interest as focus shifted to *Bd*. Recent studies into the immunocompromising abilities of *Bd*, however, have opened new questions about its relationship with *Ah* and their combined effects on a host.

In this study, I explore the relationship between infection with these two pathogens, *Bd* and *Ah*, in two amphibian species from distinct regions of the United States. I developed a novel qPCR assay to measure the microbial load of *Ah* on the skin of two anuran species, *Lithobates yavapaiensis* (N=232) and *Pseudacris ornata* (N=169), which have confirmed *Bd* infections. I use a logistic regression model to identify whether significant relationships exist between these two pathogens, disease, and death. I find that even amongst the most severely infected frogs, *Ah* is not detectable on the skin and only appears post-mortem. I therefore conclude that *Ah* is an opportunistic bacterial pathogen, scavenging on anurans only after mortality events. This research is the first known study to quantitatively assess *Ah* in amphibians in conjunction with *Bd*. While there is no causal relationship between these pathogens, future work will examine potential *Ah* infections in other organs to more fully understand the relationship between *Bd* and *Ah*.

DEDICATIONS

First and foremost, to my parents whose love and encouragement have always guided me.
For Anna Savage, for giving me this opportunity and I could not have asked for a better teacher.
For my committee, for keeping me on the right path and providing guidance and advice.
For my friends, for keeping me motivated and happy throughout this time.
For Ariel, Andrew, and Matthew, without your patience and training I would be still be lost.
Finally for all members of PHaSeD Lab for providing guidance throughout this process.

ACKNOWLEDGEMENTS

I would like to give my sincerest gratitude to anyone who made thesis possible or aided me in the thesis process. Dr. Anna Savage, without you I would not be anywhere near completing any project or attending graduate school. I am especially grateful not only for the opportunity but your guidance which has been invaluable and has not only begun to shape me a researcher but you have taught to me think in a way I had not understood. To my committee members, Dr. Kyle Rohde and Dr. Kenneth Fedorka your guidance and expertise has been invaluable in crafting this thesis. Dr. Cynthia Bayer, thank you for reaching out for me and helping me get into a research lab for without you none of this would have been possible. I would also like to thank Andrew Mason for helping guide me through my results and statistics. In addition I would also like to thank Taina Torres for expertise and map creation in ArcGIS. Lastly to my Mom and Dad for always guiding me keeping me on the correct path even when I did not have confidence in myself. For anyone at the University of Central Florida who has helped me get me to the point I am at today.

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INTRODUCTION

Amphibian Decline

Amphibians play a crucial role in their ecosystems where they act as secondary consumers: prey for avian, snake and mammalian species and predator of insects, fish, and other invertebrates (Duellman and Trueb 1986). Amphibians serve as ecological indicators of environmental health as they have permeable, glandular skin which makes them sensitive to changes in both aquatic and terrestrial ecosystems (Vitt et al. 1990). In recent decades amphibian species have been declining at an alarming rate, with 2,468 amphibian species (43.2%) experiencing some kind of decrease and 427 (7.4%) species listed by the International Union for the Conservation of Nature (IUCN) as critically endangered (Stuart et al. 2004). While the importance of amphibian conservation for ecological health is clear, the cause of their declines are, in many cases, not well understood. There are a variety of factors that negatively impact amphibian populations, including pollutants (Linzey et al. 2003), direct anthropogenic effects (Pechmann et al. 1991), habitat destruction (Phillips 1990), invasive species (Knapp & Matthews 2000), increase in UV-B irradiation (Blaustein et al. 1994), and disease (Berger et al. 1998; Daszak et al. 2000; Landsberg et al. 2013; Chambouvet et al. 2015; Martel et al. 2013). While any combination of these factors may contribute to population declines, pathogens are consistently found infecting amphibians during documented die-offs and are likely a major factor in the global loss of amphibians (Carey et al. 1999).

Batrachochytrium dendrobatidis

Batrachochytrium dendrobatidis (*Bd*) infects the keratinized skin cells of amphibians on every continent except Antarctica (Berger et al. 1998; Lips et al. 1999; Bradley et al. 2002; Makange et al. 2012). In 1999, *Bd* was established via Koch's postulates as the cause of chytridiomycosis in a captive population of the frog species, the dyeing dart frog, *Dendrobates tinctorius* (Longcore et al. 1999; Berger et al. 1998; Pessier et al. 1999). *Bd* is one of two known species in the chytrid fungal genus *Batrachochytrium*, both of which cause disease in amphibians (Berger et al. 1998; Martel et al. 2013). *Bd* was first identified as an amphibian pathogen in Central America and Australia (Berger et al. 1998) and has now been documented to cause amphibian disease and decline on every major continent (Lips et al. 1999; Bosch et al. 2001; Bradley et al. 2002; Makange et al. 2014). *Bd* can survive and replicate across a wide temperature range from 10-25°C, however it thrives at 17-25°C, high elevation and an optimal water pH of 6-7 (Berger et al. 1998; Piotrowski et al. 2004; Hagman & Alford 2015). Due to the breadth of *Bd's* global distribution and as the cause of chytridiomycosis, *Bd* is attributed to amphibian mass mortality events (MME) worldwide across a range of amphibian species. *Bd* has also caused the extinction of several species, including the sharp-snouted day frog, *Taudactylus acutirostris* (Schloegal et al. 2006). Consequently, in many studies of disease and decline in natural amphibian populations, testing for pathogens is narrowly focused on *Bd* (Greer et al. 2005; Lips et al. 2006). This leads to the possibility that more than one pathogen is contributing to the death of amphibians in some documented declines that have been attributed to chytridiomycosis.

While *Bd* is the most notorious cause of amphibian disease, other pathogens cause MMEs in amphibians. The viral genus *Ranavirus* (*Rv*) from the family Iridoviridae has been indicted in die-offs such as in *Lithobates catesbeianus* frog farms in Brazil and in another 25 historical MME (Green & Converse 2002). More recently, a *Perkinsus*-like protist was identified as the cause of death in an MME of captive *Lithobates sphenocephalus* in Georgia, United States and has been found in six countries from three continents the around the world (Chambouvet et al. 2015). Historically, the bacterium *Aeromonas hydrophila* was thought to be the cause of the majority of amphibian disease (Emerson & Norris 1905). The number of emerging pathogens with broad geographic ranges indicates that it is important to test for all known possible causes of morbidity and mortality when MMEs occur.

Aeromonas hydrophila

The bacterium *Aeromonas hydrophila* (*Ah*) is a gram-negative anaerobic microbe which can live in varied environments, specifically both fresh and brackish water with a large temperature range of 4-37°C (Hazen et al. 1978; Nygaard et al. 1970; Rouf & Rigney 1971). *Ah* has been found on every major continent and has been found to be virulent across multiple vertebrate classes including amphibians (Hazen et al. 1978; Marquez et al. 1995; El-Taweel & Shaban 2001; Ahmed et al. 1997; Nielsen et al. 2001; Cahill & Macrae 1992). *Ah* causes disease by expressing virulence factors such as aerolysin, a hemolytic toxin that destroys red blood cells and a cytolytic pore-forming toxin, which in combination are sufficient to cause 64% mortality in experimentally infected northern leopard frogs, *Lithobates pipiens* (Rigney et al. 1978).

For over a century *Ah* has been implicated in the death of anurans in causing a disease termed “red-leg” (Emerson & Norris 1905). The aptly named disease is characterized by

cutaneous erythema with other clinical signs including anorexia, swelling, edema, coelomic effusions, ulcers, sloughing, or necrosis (Densmore & Green 2007). Since 1905, there have been several accounts of *Ah* being present in morbid amphibians (Table 1).

There have been two key investigations examining *Ah* in anurans. First, in 1949 a researcher found a lake in Charleston, West Virginia and observed around 300 frogs, some showing signs of disease but many actively calling. The following day he returned and approximately two dozen remained alive. On the third day only two remained alive, one of which died in transport to the laboratory. *Ah* was isolated from the deceased frog and via the establishment of Koch's postulates was determined to be responsible for the death of approximately 300 individuals of *Anaxyus americanus* (Dusi 1949).

The second study was done in 1983 when researchers from the University of Minnesota received anecdotal information of die-offs of *Lithobates pipiens*, the northern leopard frog, across the northeast United States (Hird et al. 1983). While occurrence of dead anurans in the field was rare, live specimens were collected, taken to the laboratory and euthanized. Once euthanized and dissected the skin and gut were swabbed and in some cases, cultures of *Ah* were established. In adult frogs the prevalence rate of *Ah* isolates ranged from 20% to 58.8%. Higher prevalence was found in tadpole populations with infection rates ranging from 50% to 84.2%. However, frogs were euthanized after collection so there are no data for morbidity due to *Ah* from that study (Hird et al. 1983). Several additional studies have documented *Ah* infecting anurans (Table 1; Emerson & Norris 1905; Dusi 1949; Hubbard 1981; Miller 2008; Hill 2010). Reports of *Ah* before the mid-1990s, however, should be evaluated with skepticism as the discovery of *Bd* and *Rv* had not yet occurred. With the ability to detect *Bd* and *Rv* coupled with

the fact that all three pathogens induce similar signs of disease in amphibians, the term “red-leg” is no longer synonymous with *Ah*. *Ah* is known to be an opportunistic pathogen in both fish and humans (Allan & Stevenson 1981; Agger et al. 1984) and has been suggested that to act similarly in amphibians. Opportunistic pathogens are microbes that normally do not attack, however when the host becomes it becomes immunocompromised the opportunistic microbes can begin to cause disease in their host (Peloux 1985). A common mechanism for a host to become infected by an opportunistic pathogen is by co-infection in which one pathogen lowers the immune system and the secondary pathogen causes further damage to the host.

Table 1: Published Records of Amphibian Infection Leading to Disease that Involve *A. hydrophila*

Location	Species	Date	Stage	No. infected	Mortality rate (%)	Co-infection
Chicago, IL, U.S.	*Lithobates tigrina*	1905	Adult	4	100	Untested
Ithaca, NY, U.S.	*Lithobates esculenta*	1905	Adult	5	40	Untested
Charleston,WV, U.S.	*Anaxyus americanus*	1949	Adult/ tadpole	Roughly 300	100	Untested
Unknown, U.S	*Lithobates pipiens*	1978	Adult	55	26	Untested
14 sites in MN, U.S.	*Lithobates pipiens*	1981	Adult/ tadpole	376	N/A	Untested
Unknown	*Xenopus laevis*	1981	Adult	21	86	Untested
11 sites in MN, U.S.	*Lithobates pipiens*	1983	Adult/ tadpole	222	N/A	Untested
Georgia, U.S.	*Dendrobates auratus*	2008	Adult	3	100	Bd/Rv
Georgia, U.S.	*Phyllobates terribilis*	2008	Adult	1	100	Bd/Rv
Georgia, U.S.	*Rhacophorus dennsyi*	2008	Adult	1	100	Bd/Rv
Georgia, U.S.	*Pyxicephalus adspersus*	2008	Adult	1	100	Bd/Rv
Santiago, Chile	*Xenopus laevis*	2010	Adult	1	100	Bd/Tb

Co-infection

Co-infection is defined as the stable coexistence of different parasites, bacteria, fungi, viruses or any combination in the same host (May and Nowak 1995). The combined effects of such pathogens pose diagnostic and therapeutic challenges due to difficulty parsing out which of the pathogens is responsible for the symptoms or if the symptoms only appear when the pathogens work synergistically (Pawlowski et al. 2012). One of the concerns with co-infection is the combined effects on the host's immune system: individuals with immunosuppression caused by an infection are at increased risk of infection by a secondary pathogen that would not cause

damage in an immunocompetent host (Palefsky & Holly 2003). Primary immunodeficiency is when a host is infected for the first time and the immune system becomes compromised (Bonilla et al. 2015). A primary immunodeficient host is susceptible to secondary infections which further lowers the immune system leading to an increase in the pathogen load of both species (Slifka et al. 2013). Recent studies have documented *Bd* and *Ah* co-infecting a deceased frog host (Hill 2010; Miller 2008). *Bd* may inhibit the production of key components of the adaptive immunity in a host, which could lead to primary immunodeficiency in an anuran, thus making it more susceptible to infection and those infections increasing in load.

***Batrachochytrium dendrobatidis* Infection Suppresses the Host Immune System.**

Bd possesses the ability to suppress lymphocyte proliferation and prevent normal function of cultured *Xenopus laevis* lymphocytes *in vitro* (Fites et al. 2013). To my knowledge, no studies have been conducted where *Bd* is specifically tested as a cause of stress or immunosuppression rather than the proximate cause of mortality. Therefore, it is possible that *Bd* does not directly cause some amphibian mortality events that have been attributed to chytridiomycosis. Because *Ah* is known to be a secondary invader that infects stressed hosts who are immunocompromised in non-amphibian taxa (Majumdar 2005), and *Ah* is a documented amphibian pathogen (Emerson & Norris 1905; Hird et al.1983), one plausible scenario is that *Ah* contributes to or even causes some amphibian mortality events attributed to *Bd*. A further complication is that *Ah* has been suggested to be part of the normal flora of anurans and may be present in non-diseased animals (Miller et al. 2009). However, no previous research has directly quantified whether *Ah* is a common amphibian skin microbe in healthy individuals.

Objectives

Lithobates yavapaiensis, the lowland leopard frog, is endemic to the southwestern United States. I selected *L. yavapaiensis* for this study because *Ah* and *Bd* are both known to occur, *Bd* infects amphibians in Arizona, and in *L. yavapaiensis* MMEs occur and are attributed to *Bd* (Savage et al. 2011; Savage et al. 2015; Savage & Zamudio 2016; Silvestry-Rodriguez et al. 2007). I also targeted *Psuedacris ornata*, the ornate chorus frog endemic to the southeastern United States, because *Ah* and *Bd* have also been detected in this region but no *Bd*-associated MMEs have been documented (Horner et al. *in prep*; Esch et al. 1976; Hazen 1978). Given these contrasting regions, species and histories of MMEs, investigating these two species will enable determination of the breadth of *Ah* occurrence and potential association with *Bd* MMEs.

Here, my objectives were to (1) detect whether *Ah* is present in *P. ornata* or *L. yavapaiensis*, (2) determine if there is any relationship between the presence of *Bd* and the presence of *Ah* in individuals of these species, and (3) in *L. yavapaiensis* populations with *Bd*-associated mortality, assess whether an individual's health status is positively correlated with *Ah* infection. Few quantitative assays exist that are specific for detecting *Ah*, and none exist for amphibians. To test for the presence and quantity of *Ah* in amphibians I therefore developed a novel quantitative (q)PCR assay that reliably detects *Ah* collected from amphibian hosts. I use this novel assay to test my central hypothesis: ***Ah* infection contributes to amphibian declines in the United States, and is significantly associated with amphibians that are infected with *Bd* and manifesting disease signs.**

METHODS

Sequence Alignment and Primer Development

In order to detect the presence of *Ah*, I used polymerase chain reaction (PCR) to amplify several *Ah* loci. Several PCR primers and amplification conditions were utilized from known literature (Trakhna et al. 2009; Kingombe et al. 1999; Griffen et al. 2013), however none of these attempts were successful. I therefore designed novel primers targeting the *Ah* aerolysin gene. The aerolysin gene was selected because it codes for the virulence factor that is a hemolytic, pore-forming toxin specific to the genus *Aeromonas* (Howard et al. 1987). All available *Ah* aerolysin sequences taken from amphibians in the nucleotide database were downloaded from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/) and aligned in Geneious (BioMatters) using the ClustalW algorithm (Larkin et al. 2007). Two conserved regions in the aerolysin gene were identified as good primer locations and a combination of Primer3 (Untergasser et al. 2007) and manual primer design were used to design forward and reverse primer pairs.

Ah qPCR assay development

Ah cultures isolated from amphibians and other vertebrate taxa were donated by the University of Georgia (Athens, GA, USA) for use as positive controls. DNA was extracted following manufacturer's instructions (QIAGEN DNeasy Blood & Tissue Kit, Qiagen, Valencia, CA). Additionally, four DNA samples from *L. yavapaiensis* and *P. ornata* toe clip or skin swabs were tested using the novel primer pairs. Samples were amplified using a T-100 thermal cycler (Bio-Rad, Hercules, CA) using 10.5 ul of 1x Taq Buffer, 1 ul of forward primer, reverse primer, and dNTP, 0.5ul of *OneTaq* polymerase (New England Bio-Labs, Ipswich, MA) and 1 ul of DNA sample. Amplification was done using the following cycling parameters: Initial

denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 59°C for 30 seconds, and an extension at 72°C for 30 seconds. Followed by a final extension at 72°C for 5 minutes. Amplified products were separated by gel electrophoresis on a 2% agarose gel, visualized under UV light, and cleaned using Exo-Sap (Affymetrix). PCR products were Sanger sequenced at the Eurofins DNA sequencing core facility (Louisville, KY, USA). Sequences were compared to NCBI reference sequences of *Ah* and confirmed to have significant homology to the *Ah* aerolysin gene sequences in GenBank.

Ah aerolysin sequences from positive control and toe clip samples were then aligned and used to design a conserved internal TaqMan fluorescent probe (Eurofins Genomics, Louisville, KY) for quantitative (q)PCR. The designed primer pair and probe were then used to optimize a qPCR protocol implemented using the CFX96 Real-Time System (Bio-Rad, Hercules, CA) using Bio-Rad super mix (BioRad SsoAdvanced Universal Probes Supermix, Hercules, CA). I synthesized a gBlock gene fragment comprised of the amplified region of the *Ah* aerolysin gene (Integrated DNA Technologies, Coralville, IA) in order to generate a standard curve for quantifying the *Ah* infection intensity (number of gene copies) in unknown samples. Serial dilutions of this gene fragment ranging from $2x10^7$ to $2x10^0$ gene copies were run with every qPCR assay, along with positive controls (*Ah* culture DNA) and negative controls (deionized water). All standards and controls were run in duplicate.

The standard serial ten-fold dilutions of standard gene blocks (Integrated DNA Technologies, Coralville, IA) ranging from values $2x10^7$-$2x10^0$ were created to generate standard curves. Based on these curves, C_q values for each sample were converted into the total number of *Ah* gene copies present in each sample. C_q values are defined as the number of cycles required

for fluorescent signal released by the probe to exceed the background noise. The curves generated by the C_q values were used to make a best fit line to determine the infection intensity of the unknown samples of *L. yavapaiensis* and *P. ornata.*

Amphibian sampling

Pseudacris ornata toe clips were originally collected in summer months from three South Carolina locations and one Florida location as part of several larger population genetic studies (Degner et al. 2008; May et al. 2011; Hether & Hoffman 2012). These four populations were selected for this study because of large sample sizes (N ≥25) and variable *Bd* prevalence (Horner et al. in prep). Only live, apparently healthy frogs were sampled, although no attempts were made to characterize disease, morbidity or mortality.

Lithobates yavapaiensis skin swabs were collected using standardized swabbing protocols (Hyatt et al. 2007) during two distinct seasons, summer and winter, for disease studies of *Bd* and chytridiomycosis (Savage et al. 2011; Savage et al. 2015; Savage & Zamudio 2016). In summer months (June-September) few frogs were infected with *Bd* and die-offs did not occur. In winter months (October-March) high *Bd* loads and die-offs occur and have been attributed to *Bd*. For both the winter and summer samples, the same five geographic locations of *L. yavapaiensis* were tested for *Ah*. In the original research of *L. yavapaiensis* investigators were focused on disease, specifically chytridiomycosis, information on the health status of each individual (healthy, sick, or found dead) was collected. For populations of *L. yavapaiensis* however, a health status was observed and characterized. A healthy frog was categorized as any frog that did not exhibit any signs of disease. A sick frog was categorized as any frog manifesting signs of chytridiomycosis, including redness, lethargy, lack of righting response,

lack of escape response, and sloughing of skin. Any individuals showing severe signs of sickness were placed under observation until recovery or death, and in all cases these sick frogs died within 12 hours (Savage et al. 2011). A dead frog was categorized as any frog that was found dead.

Bd quantification

Pseudacris ornata toe clips were tested for the presence and quantity of *Bd* (Horner et al., *in prep.*) using a *Bd*-specific qPCR assay (Annis et al. 2004). For samples of *L. yavapaiensis*, the skin swabs were tested for the presence and quantity of *Bd* using a *Bd*-specific qPCR assay (Annis et al. 2004). For both *P. ornata* and *L. yavapaiensis* the infection intensity of *Bd* was measured. Infection intensity is the number of genomic equivalent (GE) units of microbe per sample.

Data analysis

From the health categories established *a priori* to this study (healthy, sick, dead) Kruskal-Wallis tests and the Chi-square test of independence were used to determine if there were differences in *Bd* and *Ah* load amongst healthy, sick, and dead *L. yavapaiensis.* I used a Kruskal-Wallis test because preliminary observation of the data showed a non-normal distribution of the infection loads. For *post-hoc* evaluation two separate Dunn's test were used as it allows for multiple comparisons based on non-parametric categorical data. The proportion of individuals in each group was used to establish a 95% binomial confidence interval for each group in a health category.

To test the relationship between *Bd* infection intensity and the ability to detect *Ah* in a sample, *Bd* and *Ah* were evaluated using a logistic regression with the glm function in R (R core

team). Raw data were first log transformed to correct for the data skew to account for the natural exponential growth of the fungus and bacterium. Prior to log transformation, all data were positively shifted by a numerical value of one unit (equivalent to one *Ah* or *Bd* cell) to avoid undefined points at log of zero. This method still preserves ranks of the data as it simply sets the lower bound of the log axes as zero. The logistic regression predicts the probability of a sample testing positive for *Ah* infection depending on the infection intensity of *Bd.* Success (designated as 1) was defined as a sample having *Ah* while the lack of *Ah* was designated to be 0.

RESULTS

Real Time qPCR Assay

I designed novel primer pairs (Table 2) that amplify a single 121 bp fragment of *Ah* from amphibians in conventional PCR. From the four amplified positive control *Ah* cultures, all had significant homology to NCBI *Ah* reference sequences. I designed a custom *Ah* probe for detecting the aerolysin gene consisting of a conserved 15-base pair sequence with a 6-FAM reporter and BHQ-1 quencher (Table 2). The optimized qPCR assay consisted of 8µl of Supermix, 2µl at 10µM of each primer, 3µl of molecular grade water, 5µl of 10µM probe, and 5µl of template DNA for a total volume of 25µl. Optimal cycling conditions were an initial 2 min at 50°C then an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 1 min and 59°C for 30 sec. The standard curve best fit line had a slope of -3.561 which corresponds to a 93% efficiency and R^2 of 0.874 (Figure 1).

Table 2: Primers and the probe for the qPCR assay. The forward or sense primer is denoted (F). The reverse or antisense primer is denoted (R). The probe is denoted (P).

Nucleotide sequence
Primers
Aero_F: TGGAACTGGACCRTMCAGCAG
Aero_R: TTGCCGGCAAACTGGCT
Probe
6-FAM-GTGCTGCGCCCGGTG-BHQ-1

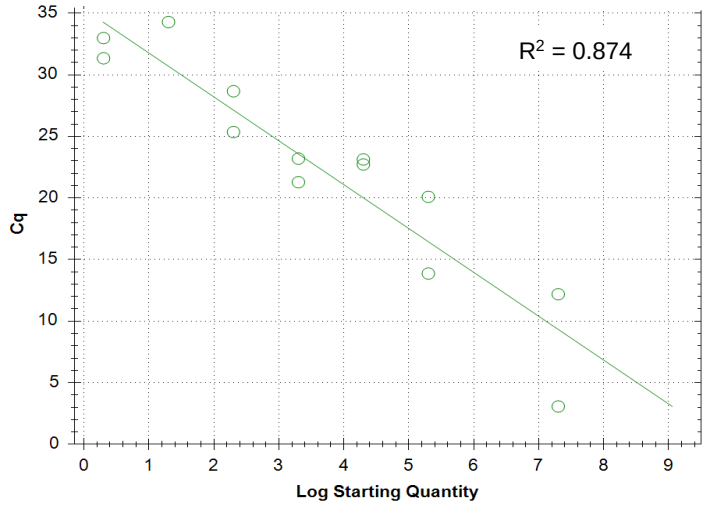

Figure 1: Standard curve of a TaqMan probe-based assay with 10-fold serial dilutions from $2.00x10^7$ to $2.00x10^0$ (circles) bacterial copies per reaction. The line is a best fit line from the serial dilution amplification. The graph of C_q is plotted against a logarithmic concentration of serial dilutions.

Analysis of *A. hydrophila* in *P. ornata* and *L. yavapaiensis*

Ah infection was not detected in any *P. ornata* population (Figure 2). Due to the absence of *Ah*, no further analyses were conducted for *P. ornata.*

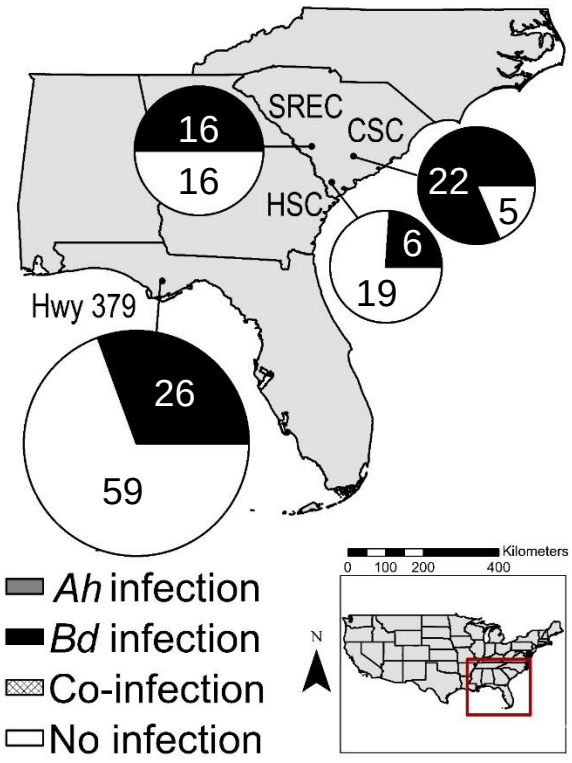

Figure 2: Infection prevalence of *Ah* (gray), *Bd* (black), co-infection (cross-hatch), and no infection (white) of *P. ornata* in the southeast of the United States. The circles are relative to the sample size of the population. The abbreviations for the populations are summarized in Table 2.

In the five populations of *L. yavapaiensis* screened for *Ah*, two were *Ah* positive in winter: Muleshoe Ranch-Bass Canyon (MRBC; five individuals) and Cienega Creek (CIC; two individuals; Figure 3). A total of four individuals were co-infected with *Ah* and *Bd* (Figure 3b). *Bd* infection intensity did not impact the probability of detecting *Ah* in a sample using a logistic regression ($p=0.419$).

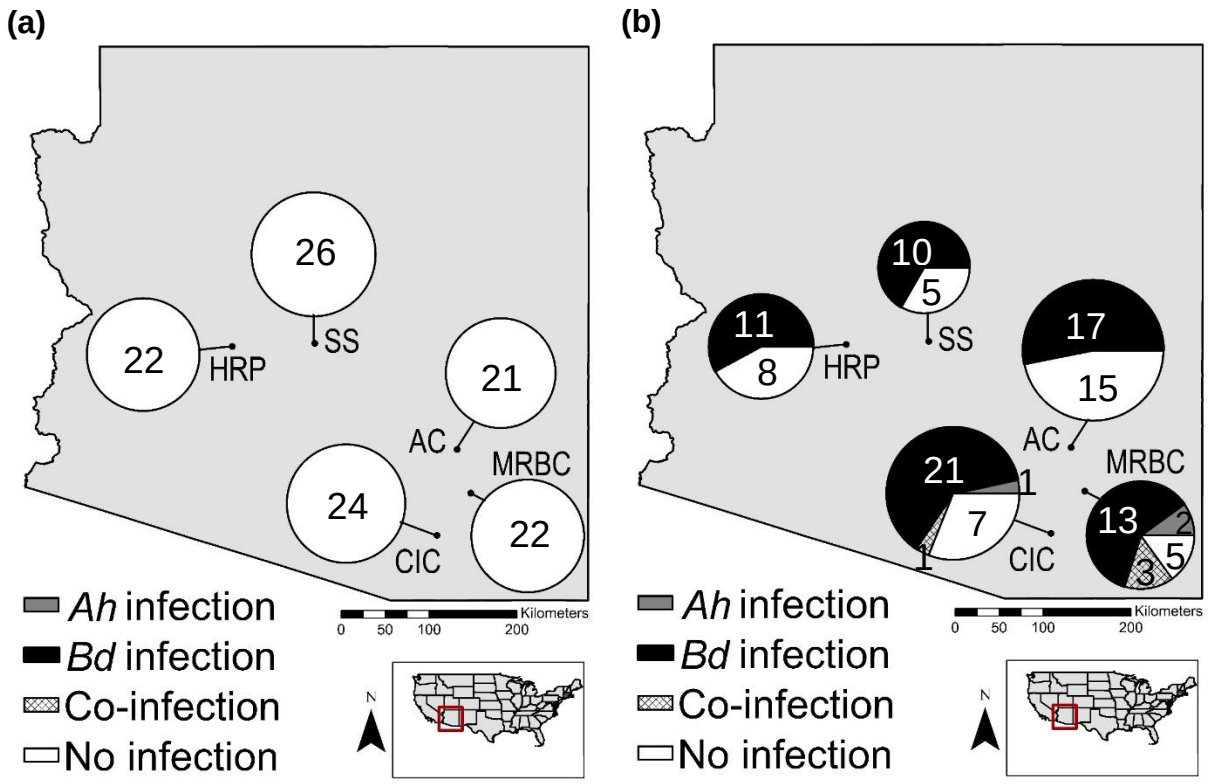

Figure 3: Infection prevalence of *Ah* (gray), *Bd* (black), co-infection (cross-hatch), and no-infection (white) of *L. yavapaiensis* in the summer (a) and the winter (b). The circles are relative to sample size of the population. The abbreviations for the populations are summarized in Table 2.

Ah was only present in *L. yavapaiensis* individuals found dead (Figure 4a). Average *Ah* infection intensity was equivalent to *Bd* infection intensity among dead frogs (Figure 4b). However, as previously determined (Savage et al. 2011, 2015), *Bd* infection occurs across all three categories (Figure 4a). The presence of *Bd* in an individual and the health status of that individual were not independent ($P = 2.417\text{x}10^{-6}$, $X^2 = 25.87$, Kruskal-Wallis test) and *Bd* infection was significantly associated with health status. *Bd* infection prevalence was not significantly different between the healthy frogs versus dead frogs ($P = 0.406$, Dunn's test). However, significantly more sick frogs were *Bd* infected compared to healthy frogs ($P <$

0.00001, Dunn's test) or dead frogs ($P < 0.00001$, Dunn's test). Similar to results for *Bd*, the presence of *Ah* in an individual and the health status of that individual were not independent ($P = 2.883 \times 10^{-5}$, $X^2 = 20.91$, Kruskal-Wallis test) and *Ah* infection was significantly associated with health status. *Ah* infection prevalence was not significantly different between the healthy frogs and sick frogs ($P = 0.500$, Dunn's test). In contrast, significantly more dead frogs were infected with *Ah* compared to healthy frogs ($P < 0.00001$, Dunn's test) or sick frogs ($P = 0.002$, Dunn's test).

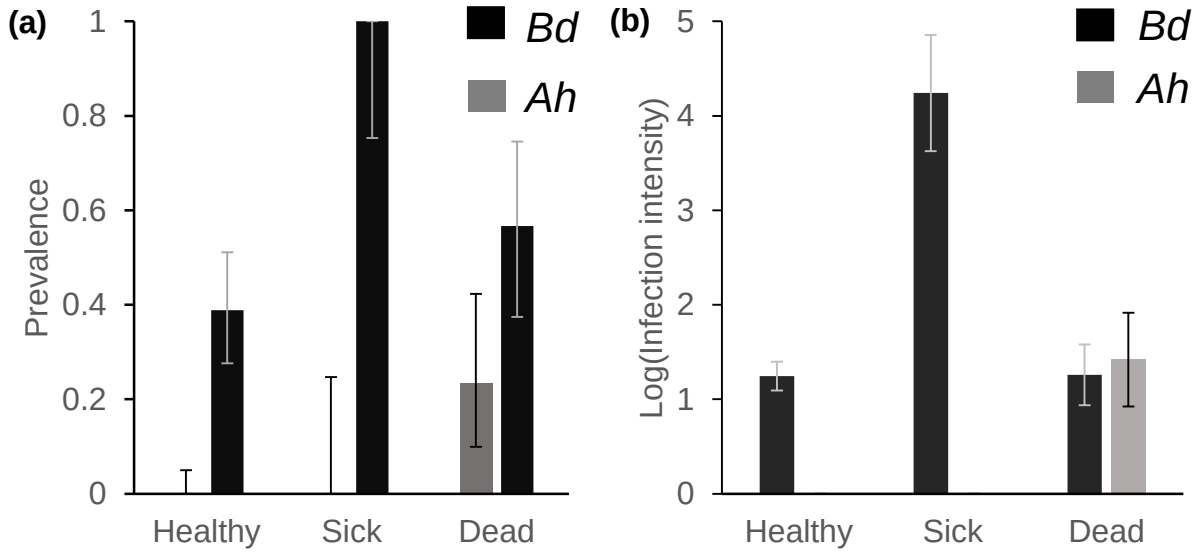

Figure 4: (a) Prevalence of *Bd* (black) and *Ah* (gray) across *L. yavapaiensis* health categories (healthy, sick, dead) in winter-sampled populations. Error bars show 95% binomial confidence intervals. (b) The infection intensity on the logarithmic scale of *Bd* (black) and *Ah* (gray) across the three categories of health states (healthy, sick, dead) of *L. yavapaiensis* samples amongst winter populations. Error bars are standard error of the mean (SEM).

Table 3: An overview of the sampling data for *P. ornata* and *L. yavapaiensis*. Populations are separated by populations with abbreviations. Summer populations are denoted (S) and winter populations are denoted (W).

Population	No. sampled	No. *Ah* positive	No. *Bd* positive
Psuedacris ornata			
Coleton, SC (CSC)	27	0	22
SREC (SREC)	32	0	16
Hardyville, SC (HSC)	25	0	6
Highway 379 (Hwy 379)	85	0	26
Lithobates yavapaiensis			
Cienega Creek (CIC) (W)	29	2	21
Aravipa Canyon (AC) (W)	32	0	17
Hassayampa River Preserve (HRP) (W)	19	0	11
Muleshoe Ranch-Bass Canyon (MRBC) (W)	20	5	13
Secret Spring (SS) (W)	15	0	10
Cienega Creek (CIC) (S)	24	0	0
Aravipa Canyon (AC) (S)	21	0	0
Hassayampa River Preserve (HRP) (S)	22	0	0
Muleshoe Ranch-Bass Canyon (MRBC) (S)	22	0	0
Secret Spring (SS) (S)	26	0	0

DISCUSSION

Bacterial *Ah* infections are seen in many taxonomic groups, including fish, porcine, equine, and humans (Austin 1996; Gray 1984; Daskalov 2006). *Ah* is capable of being present in any freshwater supply (Hazen et al. 1978). This is the first study to quantitatively measure the infection intensity of *Ah* in amphibians.

There are two major reasons why I developed a qPCR protocol to quantify *Ah* in amphibians. First, a TaqMan qPCR assay, unlike conventional PCR, uses a probe, which increases the overall specificity and the sensitivity of amplifying a particular molecular target. The second is that a qPCR assay not only detects the presence or absence of its target, it also quantifies the amount of DNA in a sample. This qPCR assay was developed to quantify the microbial load of *Ah* on any individual and seek to draw from this if there was relationship between *Ah* and *Bd* infections in a host. This assay is capable of detecting a virulence factor specific to the genus *Aeromonas*, amplifies the gene that is specific to *Ah*, and quantifies at numbers as low as two bacterial gene copies and thus can be used in identification and quantification of *Ah* in future amphibian disease studies and for amphibian health screening.

Ah is considered a ubiquitous bacterium in water, yet in *P. ornata* and *L. yavapaiensis,* which both exhibit at least a partial aquatic lifestyle as adults, there are only seven examples of *Ah* infection across a total sample size of 399 individuals and only in frogs found dead. While this confirms that *Ah* is present, it was only detected in populations of *L. yavapaiensis* where there have been observed die-offs attributed to chytridiomycosis (Savage et al. 2011; Savage et al. 2015), and I did not find any evidence that Ah infects live amphibians due to *Bd*-imposed immunosuppression. Therefore I conclude that in these five populations of *L. yavapaiensis*, *Ah*

presence on the skin does not participate in amphibian death only appearing on the skin after the individual has died.

From previous disease studies of these populations, *Bd* was found to infect *L. yavapaiensis* (*Bd* load > 0) in 64 of all sampled individuals across populations. Yet co-infection between *Ah* and *Bd* was only observed in four events. Thus this study finds that in these samples of *L. yavapaiensis, Bd* infection, regardless of infection intensity is not an indicator of *Ah* infection on the skin.

While *Ah* was detected and quantified in individuals of *L. yavapaiensis* there is no evidence in this study to support that *Ah* is the cause of their death or plays any role in amphibian mortality on the skin of these populations of *L. yavapaiensis*. The first reason for this is that there is no detection of *Ah* in any frog sampled while alive. The second reason I believe *Ah* is likely a non-factor in amphibian mortality events, yet observed on dead anurans, is the fact that *Ah* is an opportunistic pathogen only becoming present when an individual is immunocompromised or, in this case, dead.

To account for the presence of *Ah* in dead frogs, I propose a new hypothesis: whereas *Bd* inhibits lymphocyte proliferation, while the animal is still alive, the frog's innate immunity is sufficient to prevent *Ah* infection. Thus when frog die-offs occur in water and are attributed *Bd*, *Ah* rapidly colonizes the skin. *Ah* inhabits fresh water locations which are the same areas frogs which have died from *Bd* are found. *Ah* is able to act as an opportunistic pathogen because after the frog's death no immune defense exists and thus *Ah* is only then able to reproduce and feed on amphibian skin only after the frog has died.

Although I found no relationship between *Bd* and *Ah*, it is interesting to note that those populations that had *Ah* infections, MRBC and CIC, were also the *L. yavapaiensis* populations that are most susceptible to die-offs from *Bd* (Savage et al. 2011; Savage et al. 2015). It is important to continue to monitor disease when amphibian mortality events occur so all the variables can be considered when determining the cause of death.

Furthermore while few of these samples were positive for *Ah* it is possible that *Ah* is still present and thus may have a role in amphibian disease. All 399 of these samples came from skin or a toe clip, while this sampling method works well for *Bd* detection, skin or tissue may not be a reservoir for the bacteria. In both fish and humans *Ah* has been cultured from intestinal and/or kidney samples (Khalil et al. 2013; Igbinosa et al. 2012; Cipriano et al. 1984). Future work in *Ah* infection in amphibians should look at these organs either through natural experiments or experimental infections.

Conclusion

Emerging infectious diseases are important factors in animal decline including but not limited to, chytridiomycosis in amphibians (Berger et al. 1998; Lips et al. 1999), white nose syndrome in bats (Froschauer & Coleman 2012), and colony collapse disorder amongst honeybees (Oldroyd 2007). While this work found few answers as to the role *Ah* as an infectious disease in amphibians, it did lead to the creation of a new, effective qPCR assay which can detect a bacterium that is present worldwide and is found across a variety of taxa. It is important to continue monitoring these emerging diseases so that their cause can be discerned and appropriate protocols can be applied to limit the loss of these species which is vital for our ecosystems to flourish and allow these ecosystems flow in concert with one another.

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