UNIVERSITY OF CALIFORNIA

Santa Barbara

Host, Pathogen, Environment (and Reservoir, and Vector): Understanding the amphibian-killing pathogen outside of the amphibian

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Ecology, Evolution, and Marine Biology

by

Tatum Shaw Katz

Committee in charge:

Professor Cheryl J. Briggs, Chair

Professor Wendy Meiring

Professor Holly V. Moeller

Professor Hillary S. Young

June 2022

The dissertation of Tatum Shaw Katz is approved.

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June 2022

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Vita of Tatum Shaw Katz

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EDUCATION

PhD. Candidate (2022) – University of California Santa Barbara; Ecology, Evolution, and Marine Biology Department
Committee chair: Dr. Cherie J. Briggs
Committee members: Dr. Holly V. Moeller, Dr. Hillary S. Young, Dr. Wendy Meiring

Investigate potential vectors and reservoirs of amphibian pathogen *Batrachochytrium dendrobatidis* using novel laboratory methods, field work, and quantitative methods. Develop epidemiological models and utilize Bayesian, machine learning, and regression methods to describe the interactions between potential vectors and reservoirs, amphibians, and the pathogen.

M.A. in Statistics (2021) – University of California Santa Barbara;
Statistics and Applied Probability Department
Concentration: Data Science

Studied theory and applications of regression, multinomial methods, Bayesian statistics, machine learning, and big data analytical methods. Degree conferred separately but during doctoral work.

B.A. in Biology *Cum Laude* (2017) – Occidental College, Los Angeles, CA;
Concentration: Cellular and Molecular Biology
Honors thesis: "Probabilistic spatial modeling, model selection, and chytridiomycosis: changing how we plan for invasive species"
Honors advisor: Dr. Amanda J. Zellmer

Developed spatial models of spread of the invasive amphibian pathogen *Batrachochytrium salamandrivorans* using machine learning and evaluated various model selection tools for their ability to identify a best-fit model generated through the machine learning algorithm Maxent. Collaborated with the USGS Amphibian Research and Monitoring Initiative to monitor salamander populations for disease.

RESEARCH INTERESTS

Infectious disease, quantitative and statistical methods

AWARDS, GRANTS, & FUNDING

** = wrote more than ¼ of grant but not listed as PI*

- Worster Summer Research Fellowship (2021) - $6,580
- Worster Summer Research Fellowship (2020) - $5,000

- Schmidt Family Foundation Mentorship Award (2019) - $8,000
- National Institute of Health (NIH) Ecology and Evolution of Infectious Diseases grant (2019)* – (Senior Personnel; with Cherie Briggs, Renwei Chen, Michelle O'Malley, Taegan McMahon, Pieter Johnson) – *From specialist to generalist: a multidisciplinary approach to broadening our understanding of biotic and abiotic reservoirs of emerging fungal pathogens* - $2,383,000
- Institute for the Study of Ecological Effects of Climate Impacts (2018) - $1,976
- Mildred E. Mathias Graduate Student Research Grant (2018) - $2,000
- Worster Summer Research Fellowship (2018) - $5,000
- UCSB EEMB Graduate Program Research Award (2018) - $1,600
- Valentine Eastern Sierra Reserves Graduate Student Research Grant (2018) - $2,000
- Mellichamp Fellowship in Systems Biology and Bioengineering (2017) - $10,000
- Henri Seibert Award in Ecology, Honorable Mention, *Society for the Study of Amphibians and Reptiles* (2017)
- Maria Pereyra Award, *Biology Department, Occidental* College (2016) - $1,000
- Summer Research Program Grant, *Undergraduate Research Center, Occidental College* (2016) - $4,070
- Travel Scholarship, *Harvard T.H. Chan School of Public Health* (2016)

PUBLICATIONS AND PRESENTATIONS

** = undergraduate student or mentee*

Le, M.,*, Meiman, A., Covey, A., Gole, A., Meng, M., Villa, N., Litvin, S., **Katz, T.S.**, Deshmukh, R. Participation gap analysis among energy efficiency programs in California's public sector. *In prep, Energy Research and Social Science*

McMahon, T., **Katz, T.S.**, Barnett, K.M., Hilgendorff, B. Centrifugation is an effective and inexpensive way to determine *Batrachochytrium dendrobatidis* quantity in clean water samples. *In prep, Diseases of Aquatic Organisms*

Zellmer A.J., Slezak P., **Katz, T.S.** (2020). Clearing up the Crystal Ball: Understanding Uncertainty in Future Climate Suitability Projections for Amphibians. *Herpetologica* 76(2): 108-120. DOI: 10.1655/0018-0831-76.2.108.

Katz, T.S., Zellmer, A.J. (2018). Comparison of model selection technique performance in predicting the spread of newly invasive species: a case study with *Batrachochytrium salamandrivorans*. *Biological Invasions*, DOI: 10.1007/s10530-018-1690-7.

PRESENTATIONS

Katz, T.S., Zellmer, A.J. Comparison of model selection technique performance in predicting the spread of newly invasive species: a case study with *Batrachochytrium salamandrivorans.* Oral Presentation. *The 16th Ecology and Evolution of Infectious Diseases Conference* 2018, University of Glasgow, Scotland

Katz, T.S., Zellmer, A.J. Incorporating Model Selection in Predicting the Spread of Invasive Fungal Pathogen *Batrachochytrium salamandrivorans.* Oral Presentation. *The Joint Meeting of Ichthyologists and Herpetologists* 2017, Austin, TX

Katz, T.S., Meiring, W., Briggs, C.J. Can we predict chytrid outbreaks without touching frogs?: a Bayesian approach. Oral Presentation. *Ecological Society of America Annual Meeting 2021*

Katz, T.S., Wilber, M., Briggs, C.J. From specialist to generalist: a community ecology approach to understanding the disease dynamics of the amphibian-killing pathogen *Batrachochytrium dendrobatidis*. Poster Presentation. *The 18th Ecology and Evolution of Infectious Diseases Conference* 2021, Infectious Diseases Research Institute of Montpellier, France

SELECTED STATISTICAL AND QUANTITATIVE SKILLS

- Bayesian inference
- Time series analysis
- Statistical consulting
- EM algorithm
- Markov processes and related MCMC extensions
- Supervised and unsupervised machine learning (PCA, splines, random forests, hierarchical cluster analysis)
- Linear, generalized linear, and multinomial regression
- Epidemiological and immuno-epidemiological models (systems of ordinary differential equations, individual-based models, integral projection models)
- Highly proficient in R programming language (7 years' experience)
- Intermediate in Python programming language
- Beginner in Mathematica and Java programming language

SELECTED COURSEWORK

Doctoral Coursework

UC Santa Barbara, Ecology, Evolution, and Marine Biology Department

- ***Building Ecological Models****:* Developed and analyzed an ecological model of a sapronotic pathogen. Completed an oral presentation and written report.
- ***Quantitative Methods in Biology****:* Survey class of various modeling techniques including systems of ODEs, partial differential equations, equilibrium analysis, stability analysis, simulation and numerical methods, dimensionless form, and sensitivity analysis

Master's Coursework

UC Santa Barbara, Statistics and Applied Probability Department

- ***Advanced Statistical Methods***: Three courses, broken down into linear regression, GLM, and multinomial regression. Studied mathematical proofs, theory, and applications. Completed a final project for the GLM portion of the class which involved analysis and report writing of a dataset chosen by the professor. Taught in R.
- ***Bayesian Data Analysis****:* Theoretical foundations and applications of Bayesian inference. Parameter estimation, testing, prediction, and computation methods. Taught in R.

- ***Computational Techniques in Statistics****:* Survey class of computationally-intensive methods in statistics including optimization, combinatorial optimization, EM algorithm, Monte Carlo simulation, and MCMC methods. Completed a final written report using data from my thesis work in collaboration with another student. Taught in R.
- ***Time Series****:* Theory, methods, and applications. Topics included stationarity, seasonality, ARMA models, Yule-Walker estimates, ML method, diagnostics, forecasting, and spectral analysis. Completed a final written report on tree ring size over 4,000 years. Taught in R.
- ***Statistical Machine Learning***: Survey class of methods and applications including classification and regression trees, random forests, clustering, association rules, model evaluation, and comparison. Completed a final written report to explore ecological characteristics of my thesis study sites. Taught in R.
- ***Statistical Data Science****:* Overview and use of data science tools for data retrieval, analysis, visualization, reproducible research, and automated report generation. Taught in Python.

Bachelor's Coursework
Occidental College, Biology Department
- ***Immunology:*** Studied the human immune system. In lab, developed antibodies against *Corynebacterium pseudotuberculosis* for use in research. Worked with mice to produce monoclonal immortalized b-cells.
- ***Microbial Pathogenesis****:* Studied the human immune system and various pathogens and methods. In lab, conducted research to investigate the effects of various antimicrobial substances on skin swab cultures. Completed a final presentation and written report on the findings.

TEACHING & MENTORING

Associate Instructor
- ***Biometry*** (EEMB 146) – University of California, Santa Barbara (Winter 2021, Spring 2022) – 72 – 160 students. Created lecture materials, delivered live recorded lectures via Zoom and in person, held office hours, led article discussions.

Graduate Teaching Assistant
- ***Journal Club*** (EEMB 194BC) – University of California, Santa Barbara (Spring 2020 – Summer 2021) - ~10 students/quarter. Created a journal club to continue the mentoring and professional skill development of undergraduates during the COVID-19 pandemic. Organized weekly meetings to discuss literature, teaching coding and professional skills, and foster community.
- ***Biometry*** (EEMB 146) – University of California, Santa Barbara (Spring 2018, 2019, 2020, Summer 2020, 2021) – ~60 students/quarter. Responsible for leading two, two hour lab sections, grading quizzes and lab assignments, office hours, and helping design labs. Created online content for remote learning.

- ***Ecology of Infectious Disease*** (EEMB 40) – University of California, Santa Barbara (Summer 2018, Fall 2019) – 71 students. Responsible for leading two or three, hour and twenty minute discussion sections, making and grading quizzes, and office hours

Certified Instructor for The Carpentries

- ***Carpentries.org:*** Certified instructor to teach live coding and data science workshops under The Carpentries framework

Students Mentored

- 30 undergraduate students, including one through the Women in STEM Mentorship Program and one through the California Alliance for Minority Participation

ACADEMIC ACTIVITIES AND SERVICE

- Reviewer for journal Oikos (2022)
- Reviewer for journal Frontiers in Zoology (2021)
- Reviewer for journal Biotropica (2019, 2021)
- Reviewer for journal PLoS one (2019)
- Judge for 64th Santa Barbara County Science Fair (2019) – Served as a judge for the Junior High Zoology and Botany section.
- Skype a Scientist (2018-ongoing) – Skype into K-12 classrooms to talk about research and being a scientist.
- Graduate Student Advisory Committee Secretary (2018-2020) – Organize, lead, and take notes for monthly meetings.
- Women in STEM Mentorship Program (2017) – Served as a mentor to a first-year undergraduate woman in STEM.

ABSTRACT

Host, Pathogen, Environment (and Reservoir, and Vector):
Understanding the amphibian-killing pathogen outside of the amphibian

by

Tatum Shaw Katz

In an era of increase of emerging infectious diseases, fungal pathogens have historically received little attention despite their growing importance [1–4]. Fungal infectious disease emergence events began increasing dramatically in the 70s and 80s [5], and global anthropogenic change will likely cause this trend to continue. Fungal pathogens require unique methods for detection, modeling, management, and therapeutics; yet tools for understanding and mediating fungal pathogens are underdeveloped [2,4]. While different in many ways from other pathogen classes, fungal pathogens nonetheless can have similarly devastating impacts on forests, crops, wildlife, and humans [1,3,6–8]. The understudied nature of fungal pathogens coupled with a predicted rise in occurrence makes learning more about these organisms critical.

Developing tools for monitoring fungal pathogen emergence is challenging due to their unique characteristics which set them apart from other pathogen groups. Specifically, long-lived environmental stages, saprobic reproduction, and a broad host range can make detection, risk estimation, and understanding of disease dynamics difficult [6,9]. New, readily-

available molecular methods for fungal detection are recent [10], and the low number of organisms often required to induce infection is poorly matched with PCR detection limits that often require relatively high loads [11].

Batrachochytrium dendrobatidis (Bd) is a primary example of a fungal pathogen which has caused devastating global declines and for which new methods are required for detection and prediction. In my PhD thesis, I explore three questions: (1) can we detect Bd on environmental reservoirs; (2) can we predict Bd in amphibians using only information about the amphibian's habitat; and (3) can *Drosophila melanogaster* be a vector of Bd, and be used to study invertebrate-Bd dynamics? Throughout the work presented here, I demonstrate how Bd may cause variable responses in amphibian populations (i.e., epidemic vs. endemic states), and understand more about how Bd interacts with its environment as a generalist, fungal pathogen rather than an amphibian specialist.

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Chapter 1

Investigating potential environmental reservoirs of Bd: impacts of seasonality on host-pathogen dynamics

Authors

Tatum Katz[1], Renwei Chen[1], Piet Johnson[2], Cherie Briggs[1]

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2. Ecology & Evolutionary Biology; University of Colorado, Boulder; Colorado, USA

Abstract

Batrachochytrium dendrobatidis (Bd), a fungal pathogen of amphibians, is estimated to be responsible for the decline or extinction of over 500 amphibian species worldwide. As a fungal pathogen, Bd may be capable of saprobic growth and reproduction which may allow it to remain so pathogenic to amphibians despite dramatic amphibian population declines. To investigate this hypothesis, we conducted fieldwork at sites with high heterogeneity in Bd occurrence and sampled amphibians, water, soil, and nematodes to determine if soil or nematodes could function as environmental reservoirs for the pathogen. Our findings highlight the complexity in understanding Bd-amphibian relationships in a highly seasonal and ephemeral lentic system, and indicate that soil may function as a Bd reservoir in the absence of Bd positive amphibian hosts.

Introduction

In an age of emerging infectious diseases, bacteria and viruses have often taken center stage [5,9]. Yet, prevalence of fungal pathogens began increasing in the 1970's with the onset of the HIV pandemic and global anthropogenic change will likely cause this trend to continue [3,5,14]. Fungal pathogens are diverse and ubiquitous [15] and have unusual traits, including long-lived environmental stages, saprobic reproduction, and a broad host range [1]. Nearly all fungal diseases are sapronoses, a particular class of pathogens which infect a host under the right circumstances and otherwise live freely in the environment and obtain nutrients by decomposing organic material [16]. These particular characteristics not only make fungi challenging to study, but increase their ability to drive host populations to extinction and extirpation [6]. Traditional host-pathogen, density-dependent models predict that the pathogen will die out when the host falls below a critical threshold, but before the host is driven extinct – this is known as epidemic fade-out theory [17–19]. Yet, fungal pathogens may escape this fate by exploiting the traits listed above [1].

Batrachochytrium dendrobatidis (Bd) is a highly virulent, global pathogen thought to be responsible for the decline of over 500 amphibian species and the extinction of 90 [8]. Bd is a member of the fungal phylum Chytridiomycota [20], a group which are ubiquitously found in water and soil [21]. Bd is a unique member of this phyla in that most other chytrids are either saprobic decomposers or parasites of other fungi, algae, plants, or microinvertebrates [21–23]. No other chytrids are known to infect vertebrates, other than Bd and its sister species (which infects mostly urodelans), *Batrachochytrium salamandrivorans* (Bsal) [21,24]. Like many

chytrids, Bd has two distinctive life stages: zoospores and sporangia [25]. The motile, flagellated stage, termed zoospore, can move through culture, water, or amphibian skin. Once a sufficient location is reached this period of motility ends and zoospores began to develop root-like thalli while it matures into the asexual stage, termed sporangia. As the sporangia matures and grows, it produces more zoospores within itself which will release into the environment or back onto the host [25,26]. Since its formal identification in 1999, researchers have hypothesized that Bd may be a saprobe and capable of living without a host [20]. Not only would this align with other members of the phylum Chytridiomycota, but a generalist or saprobic ability may help Bd avoid epidemic fade-out and allow it to produce the high rate of extirpations and extinctions observed in the wild through two important mechanisms: 1) allowing persistence of Bd at a site in the absence of amphibians (i.e., following a die-off or between breeding seasons); and 2) allowing Bd to evolve high virulence toward amphibians without risking fade-out. In the following work, we will explore whether Bd has non-amphibian hosts and reservoirs in a system which has high seasonality of amphibian presence at the pond. The nature of this system allows us to understand if amphibians return to the pond site with Bd each year, or if Bd is maintained locally by reservoirs in the pond habitat in the absence of amphibians.

The study system of this work consists of a series of over 170 ponds in the East Bay region of California. These sites are heterogenous across a multitude of features, including land use, amphibian community, and ephemerality. Many of the ponds reside in public parks where they can be accessed by humans and their animal companions (dogs and horses) as well as free-ranging cattle. A small subset of the ponds exist within an ecological preserve

and are therefore not exposed to the public or domestic animals. Seven different amphibian species occur across these sites: *Rana catesbeiana* (American bullfrog), *Pseudacris regilla* (Pacific chorus frog), *Anaxyrus boreas halophilus* (California toad), *Taricha torosa* and *granulosa* (California newt and the granular newt, respectively), as well as two endangered species, *Ambystoma californiense* (California tiger salamander) and *Rana draytonii* (red-legged frogs). This particular amphibian assemblage is notable due to the relatively temporally-partitioned life history patterns. *R. catesbeiana* can be found in the pond year-round with the majority of their activity occurring in the mid-summer through to early winter. *P. regilla* are similarly found during most of the year with their major breeding events (where hundreds of adults return to the pond simultaneously) occurring from November to February, followed by a latent period in early spring during which eggs develop into tadpoles. Population sizes at the pond increase again in early summer as metamorphs emerge and finally decrease during another latent period in the late summer and early fall when they cannot be found in the pond habitat. *A. boreas halophilus* are relatively cryptic throughout the year with two major events at the pond habitat: breeding of adults (early winter to mid-summer); and metamorphosis of tadpoles (summer) [27]. Both *Taricha* species are typically only present at these sites during a very brief breeding event which takes place between January and March, with larvae developing throughout the summer and then quickly leaving the pond following metamorphosis, making metamorphs of these species highly cryptic. *A. californiense* similarly have a short breeding event in December through May, and post-metamorphic individuals quickly leave the pond for terrestrial habitat [28]. *R. draytonii* are also relatively cryptic in the system with adults generally only appear during short breeding events in the winter [29]. The seasonality of life history events across all

species, in summation, creates a situation in which there is high amphibian density at the ponds from November through to March (dependent on rain events), a latent period from about April to May, high density again throughout the summer, and a second latent period in the early fall. The highly seasonally-dependent nature of when amphibians utilize the pond environment makes this an excellent study system to test Bd persistence in environmental reservoirs between breeding seasons.

Another unique characteristic common to freshwater California systems is the wet/dry seasonal pattern coupled with high rates of ephemerality. Most rain events in the system occur between November and March, and last only a very short period. These rain events will fill up ephemeral ponds, which will subsequently dry out in the summer [30]. These particular sites experienced extreme drought conditions through 2012-2015, and drought continues to date [31,32]. This produced a unique natural experiment in which to test the hypothesis that Bd is maintained by environmental reservoirs in the absence of amphibians and water, as previous work has determined that Bd is not resilient to drying [33]. If Bd is found in the pond environment in the absence of both water and amphibians, that would provide evidence in favor of environmental reservoirs.

To investigate possible modes of Bd persistence in the environment in the absence of amphibians, we will focus on two possible reservoirs: soil and nematodes. One laboratory study produced findings that nematodes could be infected with and die as a result of exposure to Bd [34]. Yet, these results were quickly challenged by a second study which could not replicate the results [35]. Due to these previous and contradictory findings, we sought to

determine the ecological relevance of nematodes as a potential Bd reservoir. Because Bd is a chytrid, and given that many other chytrids are ubiquitous in the soil, we also sought to determine if soil may be a reservoir for Bd in the absence of amphibians. In the laboratory, Bd can survive in sterile river sand for up to three months post-inoculation [36], further implicating soil as a reservoir for Bd. We use eDNA detection of Bd in the water [37] as well as traditional amphibian skin swabs [38] to compare our soil and nematode findings against because Bd can be reliably detected by both of these methods.

While nematodes and soil have been shown to support Bd viability in laboratory settings, no studies exist to-date exploring the relevance of these potential reservoirs in a natural Bd-amphibian system. If Bd is truly a saprobe, then a laboratory setting can create the necessary conditions to allow Bd to survive on soil and nematodes rather than amphibians. If Bd is not found in these potential reservoirs in the natural setting, then that would provide evidence against Bd being a saprobic organism in the wild. Due to the potentially very high importance of non-amphibian reservoirs of Bd in amphibian-Bd dynamics, we sought to determine if Bd could be reliably detected on soil or nematodes, while using water samples and swabs to estimate true Bd abundance in the habitat. Specifically, we aimed to determine if Bd presence on these sources correlated to infection in the corresponding amphibian populations. We use field data from observational studies spanning two years and statistically investigate relationships between Bd detected in amphibians and Bd detected elsewhere in the environment. Interestingly, while Bd outbreaks were identified in amphibians, no water or nematode samples ever returned Bd-positive results. Perhaps of greater interest, very low Bd loads were detected only a few times in soil

samples, none of which during a time in the year when amphibians were infected with Bd at the sites. Our findings highlight the complicated relationship between Bd, its potential reservoirs, and the seasonality of both the pond environment and the amphibian life histories.

Materials and Methods

Field Surveys

Fourty-eight ponds in the East Bay region of California were sampled repeatedly between May 2019 and June 2021 in order to collect information on Bd occurrence across amphibians, soil, water, and nematodes. Ponds were either on an ecological preserve (Blue Oak Ranch Reserve, 8 sites) or in public parks (40 sites). Ponds varied greatly in size from small lakes, ranging in diameter from nearly 300 meters to as small as 20 meters when full of water. Many ponds are artificially deepened while others are natural and vernal, drying out each summer. Drought is an important factor in this system, beginning in 2012 and lasting through the end of sampling. During the worst of the drought during sampling, as many as half of the sites sampled were completely dry with a few sites having less than a few inches of water (June 2021). This study system, especially the amphibians and their parasites, have been studied intensively since the mid-2000's [30,31,39], yet no thorough investigation of alternative reservoirs of Bd has been completed. Furthermore, nearly all of the work on these specific sites occurred during the late spring through summer

(approximately May through August), therefore we chose a year-round sampling approach to investigate Bd maintenance in the environment between active amphibian seasons.

Ponds were selected with a block design of five ponds within each of five properties (Figure 1). Samples were only collected once or a few times at 28 sites during the spring and summer of 2019; however 20 focal ponds were sampled at least nine times per site, approximately every month during the summer and every other month during the rest of the year, from January 2020 to June 2021. Across all sites, there was a total of 254 unique site-date sampling events. At each site visit, amphibians were swabbed, and water, soil, and nematode samples were collected. All equipment including dipnets and boots were disinfected by scrubbing in quaternary ammonium compound 128 (diluted to 0.008% or greater [33]) between each site visit.

Amphibian Swabs

Amphibians were captured using a fresh pair of nitrile gloves or pond-rinsed dipnet. At each site visit, we attempted to capture up to ten individuals of each species-life stage combination of the following species: *R. catesbeiana*, *T. torosa*, *T. granulosa*, and *P. regilla*. If no amphibians were captured at the site, environmental sampling proceeded as described below. Frogs and toads (anurans) were swabbed (MW113, Medical Wire and Equipment Co.) 30 times as follows: five times each on each hind foot, inner thigh, and each drink pouch (Hyatt et al. 2007). Newts (urodelans) were swabbed five times on each foot, five times on the throat and venter, and five times on the cloacal-caudal area. We also recorded the species, life stage, and sex (if able), as well as weight and snout-to-vent length. Animals

were then released at the point of capture. Swabs were stored dry and on ice in the field and during transport, and in -8°C freezers in the lab until processing. Genomic DNA was extracted from swab samples using PrepMan™ Ultra Sample Preparation Reagent (Life Technologies, Carsbald, CA) (Hyatt et al. 2007) and further cleaned using GeneReleaser according to the manufacturer's instruction (BioVentures Inc., Murfreesboro, TN).

Soil Samples

Soil was collected from three different blocks around the pond: (1) the very edge of the pond so the sample was partially in the water (pond); (2) within the area of damp soil around the pond but above water (wet); (3) the region of very dry soil at least one half meter up to three meters from the wet ring, however this was found to have no effect on results and therefore results are pooled across distance treatment. Up to three samples (one from each distance treatment) per pond were analyzed in triplicate, and results were averaged within the distance treatment. Samples were collected using a fresh nitrile glove or inverted sample collection bag for each of the three areas and stored at 4°C prior to processing, up to one year post-collection. In the laboratory, soil samples were sterilely aliquoted into 0.25g samples and was used for DNA extraction using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN INC 12855-50) according to the manufacturer's instruction. DNA was stored at -20°C until further analysis.

Water Samples

Approximately one liter of water was collected from each of the 20 focal ponds (if the pond had water) during each sampling event from January 2020 through June 2021. Clean bottles and fresh gloves were used for each sample. Samples were stored at -8°C or colder until processing. In the lab, samples were vacuum filtered through as many sterile 0.22um filters (Sterivex-GP Pressure Filter Unit, Millipore SVGPB1010) as it took to reach a minimum filtration volume of 300ml, however this was not possible for some samples due to large amounts of particulate matter in the water. Filters were filled with Longmire's solution and stored at -8°C until DNA extraction. Total DNA was extracted using the Quick-DNA Miniprep Plus kit (Zymogen D4069) and replicate filters were pooled in the DNA elution step.

Nematode Samples

At each site, four samples of decaying organic material were taken from different locations around and in the pond haphazardly as material was available. Samples were kept at room temperature until further processing (1-5 days). In the lab, samples were weighed and nematodes were isolated from them using a Baermann funnel [40] over the course of 3-4 days. The Baermann funnels consist of plastic funnels connected at the bottom to a flexible tube which is pinched shut with a large binder clip to hold water inside. A kimwipe (Kimberly-Clark Professional™ 34155) is then placed into the funnel, and the decaying organic material is placed over the top. Water is added to fill the funnel and replaced as needed to prevent drying. The Baermann funnels produce an exudate of water, small bodied organisms, and nematodes which can be collected by removing the binder clip and allowing

the exudate to flow into a collection tube. Funnel exudate was stored at -8°C until samples were gently thawed and individual nematodes were sterilely pipetted out of the exudate by hand, with the goal of collecting 10 nematodes per sample. Collecting all 10 nematodes was not always possible, as many samples had no nematodes at all; similarly, some samples contained more than 10 nematodes (mean number of nematodes per PCR reaction = 8.142, median = 10). Nematodes were stored at -8°C until processing. Nematodes were homogenized using 0.5mm zirconium/silica beads and a Mini-Beadbeater, then DNA was extracted using PrepMan™ Ultra Sample Preparation Reagent. DNA concentration was measured using a Nanodrop Spectrophotometer ND1000 to ensure there is DNA in the extracted sample. DNA samples were stored at -20°C until PCR analysis.

Quantification of Bd using real-time PCR

Quantification of Bd DNA was analyzed using real-time, quantitative PCR (qPCR) with an ABI7300 Sequence Detection System (Applied Biosystems). SensiFAST Probe Hi-ROX Kit (Meridian Bioscience) was used as master mix for the PCR reaction. Primers and probe have been described in [38]. DNA extracted from nematodes, soil, and water was further diluted in water at 1:10, 1:5, and 1:5, respectively. Five uL of diluted DNA was used in total 25 uL of PCR reaction. Additionally, TaqMan™ Exogenous Internal Positive Control Reagents (Appliedbiosystems by Thermo Fisher Scientific) were included in the PCR reaction to rule out any inhibitor present in the DNA samples. Laboratory cultured Bd zoospores served as standard control and results are reported in zoospore equivalents (ZE).

Statistical Analysis

To answer the question of whether soil or nematodes could be reliable predictors of Bd infection in amphibians, we used two analyses to test (1) prediction of probability of infection of Bd on a single individual of any amphibian species from a given site and (2) prediction of zoospore load, given the animal is infected. We split the analyses in this way instead of using a hurdle model, zero-inflated model, or quasi-model to increase interpretability. In addition to these variables, we included the species of the amphibian swabbed and Julian day the sample was taken (as a third order polynomial). Pond ID, pond block, and year the sample was taken were evaluated for use as a random effect in both analyses to control for the autocorrelated nature of our sampling design. Specifically, we tested whether the presence or absence of Bd was significantly different across pond ID, pond block, or year using a Chi-square test of independence and tested whether median Bd load on a single amphibian was significantly different across pond ID, pond block, or year using a Kruskal-Wallis test. Variables which were found to be significant in these preliminary analyses were included as random effects in the regression models. Soil, nematode, water, and Julian day variables were scaled and centered for analysis.

To determine if load of Bd in soil or nematodes could predict probability of Bd infection in any amphibian species at a given site (question 1), a suite of generalized linear mixed-effects (GLME) models with Binomial likelihoods were fit to the data using the R package lme4 [41]. To determine if load of Bd in soil, water, or nematodes could predict the load of Bd on an infected amphibian (question 2), a suite of linear mixed-effects (LME) models were similarly fit to the data using the R package lme4 [41]. Only swabs which had a load greater

than zero were used for the analysis of Bd load. Bd load across replicate samples of water (replicate filters), nematodes (four samples each of approximately ten nematodes), and soil (triplicate 0.25g samples) were summed to create a single value describing Bd load in the given substrate for each pond. For both analyses, in addition to a full model using all variables described above, a reduced model (using only soil load, nematode load, and the random effects) and a null model (using only amphibian species, Julian day as a third-order polynomial, and the random effects) were fitted. Model selection across candidate models for each question proceeded by calculating AIC, BIC, deviance, and running a Chi-squared likelihood ratio test. Additionally, we performed backward model selection from each full model to identify a single best-fit model for the data. Model fit was evaluated using area under the receiver operating characteristic curve (AUC) [42] for the GLME model and by R^2 [43] for the LME model.

Results

Field Surveys

We collected swabs, nematodes, and soil from the 48 sites a total of 254 times between May 2019 and June 2021. Due to many of the ponds drying throughout the study as a function of the natural ephemerality of the system compounded with drought (18% of focal ponds were dry on site visits January 2020 to June 2021), water was collected on only 61 sampling events. 1957 amphibians of the species *Pseudacris regilla*, *Bufo boreas*, *Rana catesbeiana*, *Taricha torosa*, and *Taricha granulosa* across the 48 sites were tested for Bd (Figure 2). A total of 571 nematode samples, 61 water samples, and 191 soil samples were

collected across all sampling events and tested for Bd. Bd in amphibians was detected on 102 of the 172 sampling events where frogs were present, for a total site-level occurrence of 59.3%. Bd was more commonly found on amphibians when ponds had water (98% of Bd positive amphibians), although was still detected on amphibians at dry ponds (2% of amphibians sampled).

Bd occurrence in reservoirs

Bd was detected in soil but never detected in nematodes or water (Figure 3). Only three site visits produced a Bd-positive soil sample, site "Ba" and "Bo" in June 2019 and site "G5" in October 2020, for a total sampling event Bd occurrence in soil of 2.3%. The Bd-positive soil samples had average loads of 11.29, 1.51, and 0.62 zoospore equivalents per 0.25g of soil, respectively. Bd positive soil was only detected when ponds had water, but could be detected in the absence of Bd-positive amphibians.

Can soil, water, or nematode Bd load predict Bd presence or load in amphibians?

Water and nematode data were not included in any of the models as all samples were negative for Bd. Soil could not be used to estimate Bd load as soil was never positive when amphibians were also positive. All three candidate Bd presence models successfully converged and residual diagnostics indicated GLME assumptions were met. All model selection metrics identified the same best model for prediction of Bd presence: the null model containing only the amphibian species, Julian day as a third-order polynomial, and the random effects of pond, block, and year (Table 1).

What are the best predictors of Bd presence and load in amphibians?

Since water, soil, or nematode Bd loads were not found to be predictive for either Bd presence or load in amphibians, we sought to determine the most predictive models among the variables available. Following the same model selection framework described above, we identified a Binomial GLME which best predicted probability of Bd positive amphibians and an LME which best predicted amphibian Bd load (Figure 4). The best model to predict the probability of a Bd positive amphibian included the amphibian species, Julian day as a third-order polynomial, and the random effects of pond ID, pond block, and year of sampling. Boundary tolerance for this model was increased to $1x10^{-3}$ to allow model convergence. The best model to predict Bd load on an amphibian given the amphibian was infected with Bd included the species of the amphibian, Julian day as a third-order polynomial, and the random effects of pond ID and pond block.

Amphibian species was a significant predictor of both load and probability of Bd presence (Figure 4). *R. catesbeiana* had a significantly higher probability of being Bd positive than all other species ($z=4.399$, $p=0.00000109$), and when infected, also had significantly higher loads than all other species ($t=2.433$, $p=0.0445$). *Taricha* spp. had significantly lower Bd loads than all other species ($t=-2.32$, $p=0.02613$), yet did not have a significantly lower probability of being Bd positive than others ($z=-1.667$, $p=0.955$).

Julian day as a third-order polynomial, was a significant predictor of both load and probability of Bd presence (Figure 4). Yet, relationships differ across species and block. *P.*

regilla tend to have higher loads in the spring and early summer and loads decrease as summer transitions to fall. *R. catebseiana* generally also decrease loads through the fall, while *A. boreas halophilus* appears to increase loads at this time of year. Little data exists for *Taricha* spp. except during the late winter/early spring when they are breeding, and the limited data shows a weak decreasing trend during that time period. *Taricha* spp. and *A. boreas halophilus* seasonal trends are challenging to estimate due to low observational numbers and highly seasonally-dependent occurrence at the ponds.

While best-fit models were successfully identified, model predictive ability was generally moderate. For the model which predicts the probability of a single amphibian being Bd positive at a given site, AUC was calculated to understand how well the model can discriminate between Bd positive and Bd negative amphibians [42] and was found to be 0.81, indicating fair-to-good predictive ability of the model. For the model which predicts Bd load given the amphibian is infected, the R^2 value was found to be 0.38, indicating the best fit model describes 38% of the variance in the data.

Discussion and Conclusions

In the work presented here, we systematically sampled the environment repeatedly throughout the year in order to understand how Bd may or may not persist in the pond environment during and between important amphibian life history events such as breeding of adults and emergence of metamorphs. We found the highest prevalence of Bd on amphibians during important life history events in all species, yet found Bd-positive soil both during and between these events. Importantly, our findings show the complexity of the

study system and illustrate the interactions between Bd, the ephemeral nature of the ponds, seasonality, and amphibian species. We detected Bd in the soil at just three sampling events, yet notably these samples were collected at times when either no amphibians were present, or amphibians were present but not Bd positive (Figure 3). Furthermore, we did not detect any Bd in the pond water or in nematode samples at any point throughout our surveys. Our analysis revealed that the amphibian species and Julian day are significant predictors of Bd presence and load on amphibians, while soil was not a good predictor (Figure 4).

Throughout our surveys we detected Bd across all pond blocks and species, as well as at both dry and wet ponds (Figure 2). Our analysis indicated that *R. catesbeiana* is not only more likely to be infected with Bd, but produce swabs with significantly higher zoospore loads than other species surveyed. This contradicts previous findings that *R. catesbeiana* is tolerant to Bd infection [44,45]. We also found that *Taricha* species in our system, when infected, have significantly lower Bd loads than other species. As little is known about newt responses to Bd, our results further confirm that *Taricha*, at least in this study system, do not seem to be greatly affected by Bd [46]. *A. boreas halophilus* was also previously determined to be susceptible to Bd [45], and our finding of post-metamorphic individuals with high Bd loads (Figure 4) supports this.

We believe we detected at least one potential die-off event among *R. catesbeiana* in the late summer and early fall of 2020, specifically at a site within the Blue Oak Ranch Reserve block. Many lethargic individuals were observed along the banks rather than in the water where they typically are and most individuals had moderate to high Bd loads (mean

zoospore load per swab = 655.02 zoospore equivalents, maximum = 9325.92 zoospore equivalents, standard deviation = 1490.8 zoospore equivalents). However, Bd was not detected in the water, soil, or nematodes of the site during the outbreak event. One would expect that, during such an outbreak, Bd would be easily detectable in water[47] yet our findings do not support this and suggest that more complicated mechanisms involving detecting Bd in the environment may be at play than previously considered. Unusually, this outbreak was almost exclusively among *R. catesbeiana* who are typically not found to have high Bd loads and are thought to be tolerant to infection. Similar high-load Bd outbreaks were observed in *R. catesbeiana* in another pond block, Pleasanton Ridge Regional Park, at the same time (Figure 2), yet frogs at these sites were not obviously lethargic or suffering any symptoms of Bd. This unusual pattern could be due to a new strain of Bd which is able to cause symptoms in *R. catesbeiana*.

While most of the species studied were not frequently found at dry ponds, *P. regilla* were consistently found at dry sites throughout all pond blocks (Figure 2). Bd is not tolerant to drying [33], yet we captured *P. regilla* at sites with little to no water, in seasons with low host density. This may indicate that Bd is more tolerant to desiccation than previously thought, or that amphibian-to-amphibian transmission is sufficient to maintain Bd at a site in the absence of water.

Perhaps most interestingly is the finding of Bd positive soil across three sites in the absence of Bd positive amphibians (Figure 3). Three samples from sites “Ba” and “Bo” were run in triplicate while only one sample from “G5” was run in triplicate. All samples from

"Ba" were positive for Bd, while only one of from site "Bo" was positive. This result indicates that there may be high detection error within soil samples. One weakness of the soil sampling methodology is the very small amount of soil used per reaction; only 0.25 grams. A 10g kit is available and, in a pilot analysis, was compared as a single run to the 0.25g kit run in triplicate and no difference was found in estimated Bd load, therefore the 0.25g kit run in triplicate was used. Methods to increase the amount of soil assayed would likely highly benefit soil-based Bd detection, and the low amount of soil used is almost certainly a key factor in the low number of Bd positive soil samples. Our findings nonetheless indicate that soil may not only be an ecologically relevant reservoir for Bd, but may contribute to Bd persistence in the pond habitat when no Bd positive frogs are present.

The results of our water analysis are in conflict with previous work which demonstrated that Bd could reliably be detected in water with large enough sample size [37,47–49]. Unusually, despite Bd occurring on amphibians at relatively high prevalence and load, all of our water samples tested negative for Bd. While sufficiently large sample sizes were not possible to obtain for every site, many sites had sufficiently high water volumes and were collected at the same time as Bd positive amphibian swabs. We hypothesize that our results may differ due to pond conditions, but further laboratory experiments are needed to support this. A 2015 review of eDNA methods found that eDNA from the target organism becomes undetectable in as little as 0.9 days following presence of the organism and is affected by pH, UV exposure, temperature, and bacterial community [50], yet all field samples were frozen within this timeframe in an attempt to preserve Bd eDNA. Storage of water samples pre-filtration may have also affected detection probability, yet our storage methods are used with

success by others in the literature [51,52]. While we stored samples for over one year in some cases due to the COVID-19 pandemic, little guidance exists on storage duration and therefore we are unable to determine if this affected results. Future studies should further investigate habitat and pond parameter effects, as well as duration of sample storage, on eDNA detection probability of Bd.

All nematode samples tested negative for Bd. While previous peer-reviewed work [53] demonstrated that *Caenorhabditis elegans* can be infected with Bd, our results indicate that wild nematode populations may not be similarly affected, or at least not with high enough prevalence to be detected by our methods. This sheds important light on the difference between laboratory-induced infections and the ecological relevance of such processes. Future work exploring the minimum necessary inoculation load to induce infections in *C. elegans*, or better yet, in wild nematode populations, will help to elucidate this difference.

Ultimately, our findings demonstrate the system-unique interactions between amphibian species susceptibility, drought and ephemerality, and Bd persistence in the environment. Notably, while Bd positive soil was detected at three sites and frequently on amphibians, Bd was never detected using traditional, filtration-based eDNA methods or with a novel nematode sampling approach. Due to the few positive soil samples we have developed two hypotheses: (1) Bd occasionally found on the soil represents transient Bd spore shed from an infected amphibian (who was missed during amphibian sampling) which recently came into contact with the soil; and (2) due to imperfect detection and low concentrations, Bd was found less frequently in soil than it truly occurs. Soil analyses may prove more indicative of

true Bd presence and load on amphibians if detection error is reduced. Therefore, future research should aim to disentangle these two hypotheses, likely through the use of mesocosm studies where the environment can be more greatly controlled to reduce detection error. This is an important distinction to make because model predictions for Bd spread dynamics will change if Bd does or does not persist in soil, water and other hosts. While prior pilot analyses investigating detection probability in artificially inoculated soil, water, and nematodes produced very promising results, these results did not translate to comparison against amphibian swab data. Laboratory or mesocosm experiments could be leveraged to further determine if these reservoirs play important roles in Bd persistence in the environment or Bd infection of amphibians.

Figures and Tables

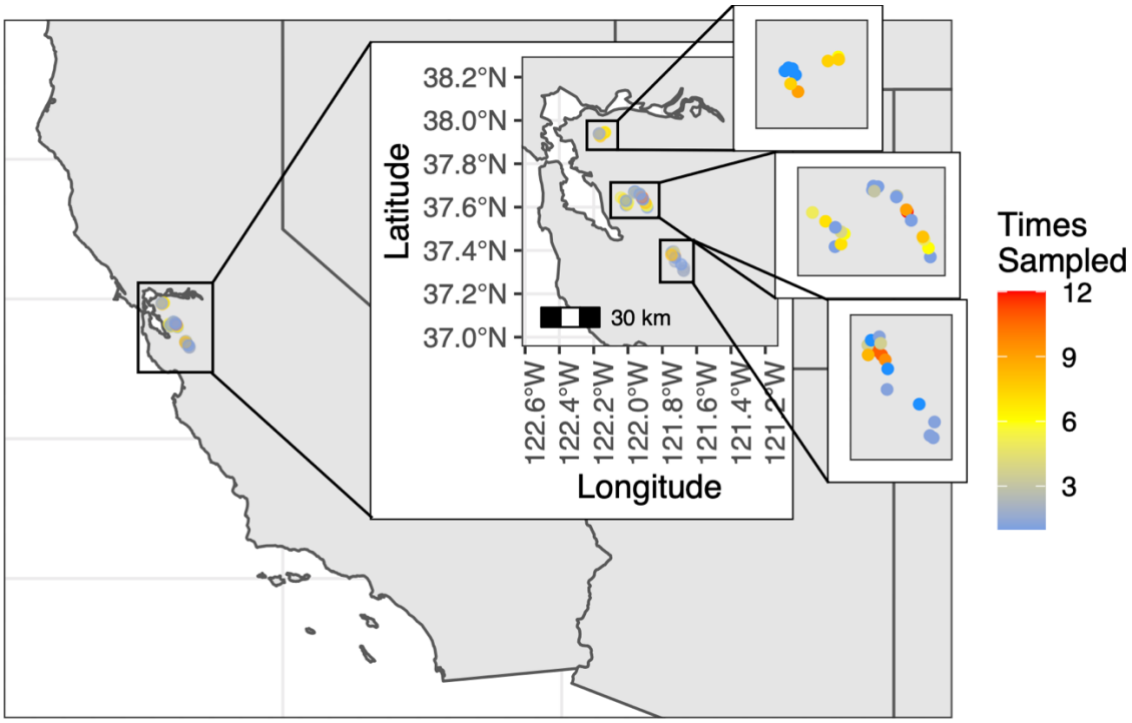

Figure 1. Each point represents a single site, while color indicates how many times that site was visited. Sites were sampled periodically from June 2019 to June 2021.

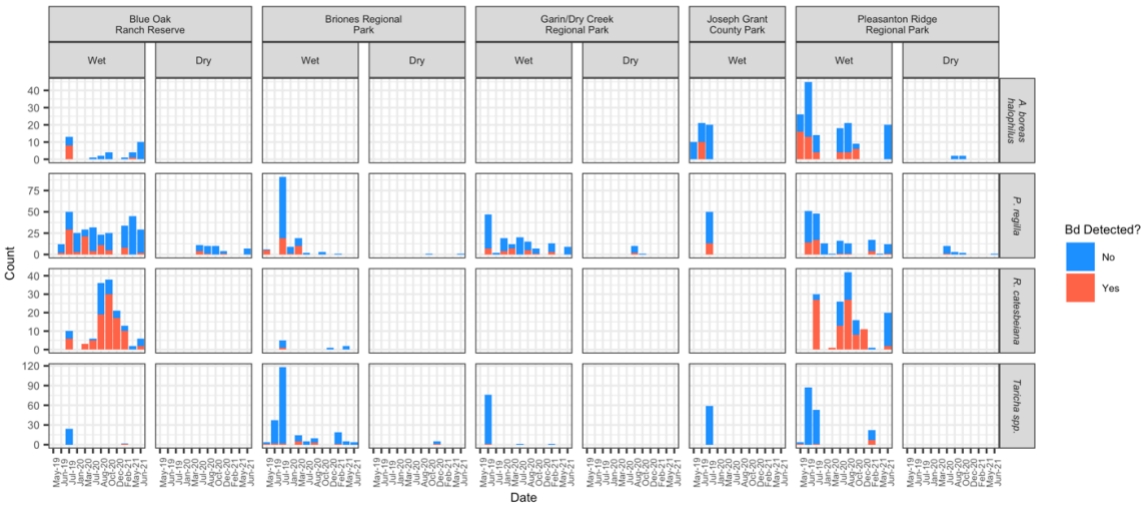

Figure 2. Bars represent numbers of swabbed amphibians of the given species at each block of sites, separated by whether those sites had water (Wet) or were dry (Dry).

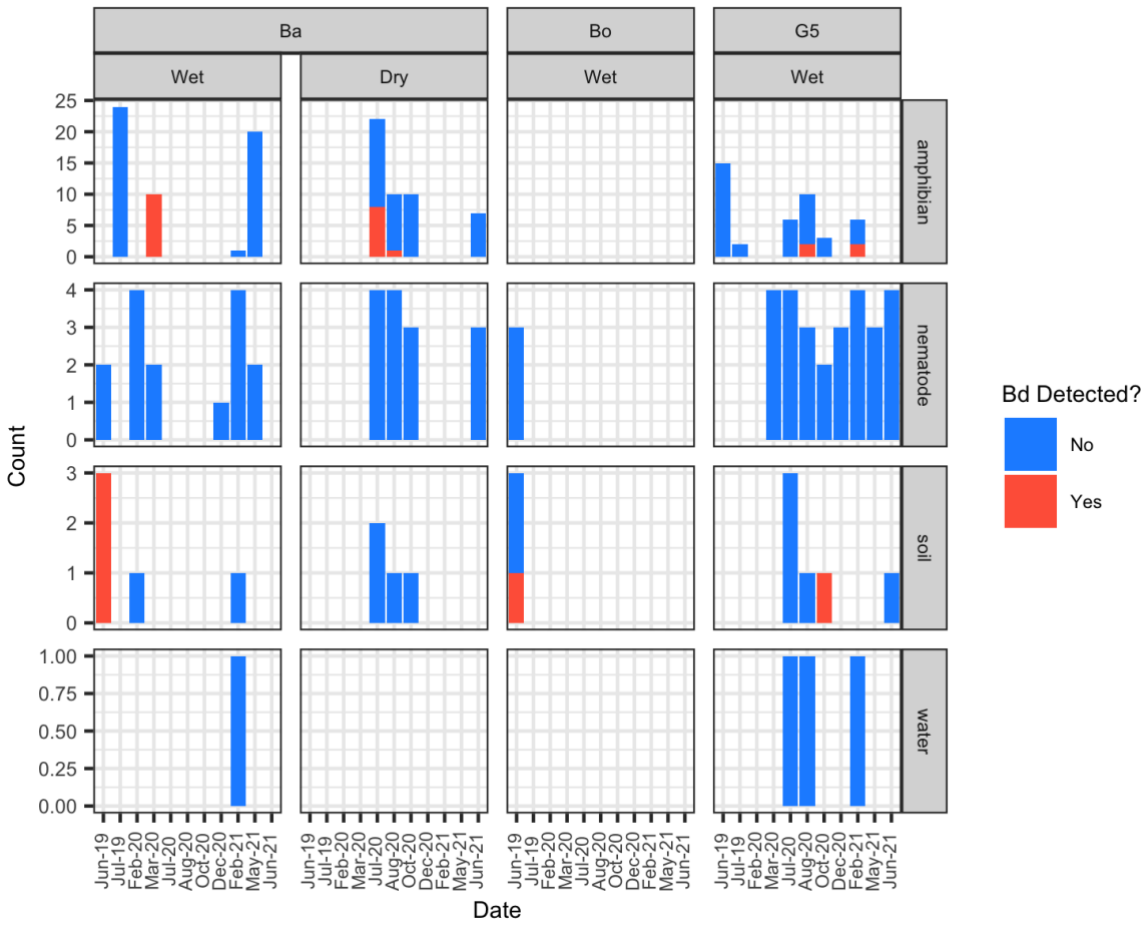

Figure 3. Bars represent number of samples assayed for Bd presence for each type of sample, for the three sites where Bd was detected in the environment (soil), and whether those sites had water (Wet) or were dry (Dry) through time.

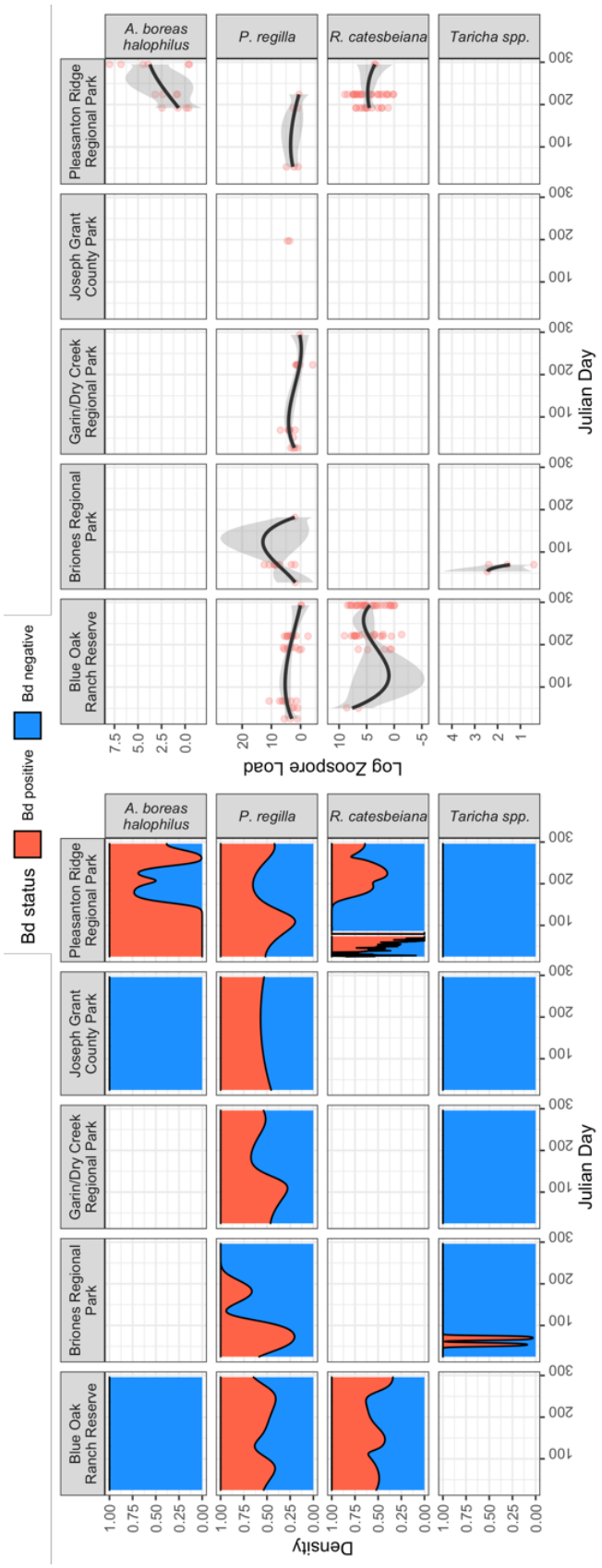
Bd status
Bd positive
Bd negative
A. boreas halophilus
P. regilla
R. catesbeiana
Taricha spp.
Pleasanton Ridge Regional Park
Joseph Grant County Park
Garin/Dry Creek Regional Park
Briones Regional Park
Blue Oak Ranch Reserve
Density
Log Zoospore Load
Julian Day

Figure 4. Plots describe results of the statistical analysis which found that amphibian species and Julian day as a third order polynomial were the best predictors of Bd presence (left) and load (right) on a single amphibian at a given site. Pond block and species combinations with less than two data points were omitted from the figures.

Table 1. Model selection of candidate models to determine what variables are predictive of Bd presence amphibians.

Model	Predictors	Number of Parameters	AIC	BIC	χ^2	χ^2 p-value
Soil Model	soil Bd load, random effect of pond, block, and year	5	746.07	768.66	NA	NA
Null Model	amphibian species, Julian day^3, random effect of pond, block, and year	10	674.55	719.74	81.51	0.000
Full Model	soil Bd load, amphibian species, Julian day^3, random effect of pond, block, and year	11	674.96	724.67	1.60	0.206

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Ethics Statement

This study and its methods were approved by the University of California Santa Barbara's Animal Use and Care Committees (protocol No. 735.3). Permission to collect *P. regilla*, *B. boreas*, *R. catesbeiana*, and *Taricha spp*. was granted by the California Department of Fish and Wildlife (permit Nos. SC-010167 and S-190450003-20052-002). Permission to perform research at specific field sites was granted by the East Bay Regional Park District (research permit No. 19-1018) and the University of California Natural Reserve System (application No. 40947).

Chapter 2

Environmental predictors of *Batrachochytrium dendrobatidis* in a California vernal lentic system: implications for management

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Abstract

Batrachochytrium dendrobatidis (Bd) is a lethal fungal pathogen affecting amphibians worldwide. Detecting Bd outbreaks is critical for management and understanding of this pathogen, and traditionally proceeds by non-invasively swabbing an amphibian and using quantitative polymerase chain reaction (qPCR) to estimate Bd load on the animal. However, swabbing amphibians comes with many challenges including handling wild animals, training personnel, and cost. Therefore, we sought to determine if Bd occurrence and load could be reliably predicted in our system using only environmental variables, with a secondary goal of identifying possible new modes of managing the pathogen. Using field-collected data along and a Bayesian modeling approach, we were able to determine many

predictive factors of Bd in the system, including water temperature, water salinity, and the presence of cattle. While the model does not successfully predict Bd events, our results identify new possibilities for management strategies in this system

Introduction

Since the early 1980's, scientists have noted the global, rapid decline of amphibians [54,55]. Explicitly human-driven processes such as habitat loss and overexploitation have historically been responsible for the majority of these declines [54]. Skeratt et al.[56] proposed that the majority of the remaining, "enigmatic" declines were due to the novel amphibian fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Since then, further evidence has only confirmed Skerratt et al.'s claim and declines have worsened, resulting in the greatest documented loss of biodiversity to a single pathogen [8,57]. In a recent review, Scheele et al. estimated that Bd is responsible for the decline of at least 500 amphibian species and the extinction of 90 [8].

Traditional Bd detection involves non-invasive swabbing of the skin of amphibians followed by DNA extraction and PCR [38]. The necessity of capturing an amphibian to detect Bd presents complications: what can be done during seasons in which the amphibians are hibernating (i.e. *Rana muscosa*), or for life stages which are extremely cryptic (i.e. non-reproducing adult *Anaxyrus boreas*)? Bd-amphibian dynamics are still at play even when researchers are not able to estimate them, and our understanding of the full system is hindered by this missing information. Additional barriers to traditional swab-based detection include securing permits (especially for endangered species, which are usually of high

interest), training personnel, and sampling enough individuals. While swabbing is a safe and non-invasive method [38], the capture and handling of already infected individuals is an unnecessary stress increase that would be beneficial to eliminate.

Due to these challenges, in recent years interest in environmental DNA (eDNA) detection of Bd has grown. While eDNA presents exciting new possibilities for amphibian-free Bd detection (i.e., [47]), it is not without limitations: difficulty in methods and high cost [58], inconsistent results compared to swab-based detection [49], variance in efficacy due to extraction method [48], and requirement of relatively advanced modeling techniques to overcome detection error [59]. Another promising, novel technique involving a lateral-flow assay and monoclonal antibody also has issues of high cost and off-target detection of another closely related fungus [60]. Ultimately, while highly useful in many situations, all of these detection methods require highly trained personnel and are often cost-prohibitive, especially for necessarily large sample sizes to overcome detection error issues. This introduces undesirable outcomes of unequal access in Bd research.

Being able to predict Bd occurrence and severity without handling animals has further benefits in the identification of areas of risk and refuge for amphibians without necessitating the need to travel to the location for amphibian sampling. Correlative techniques involving environmental variables applied to conservation concerns have revealed valuable insights [61], and these techniques have been applied to amphibian conservation questions with success in identifying potential refugia from Bd [62–67].

In light of these issues of access and cost, and the high potential benefit of understanding environmental drivers of Bd across a system, we sought to determine if we could predict Bd infection in amphibians by only measuring relatively low-cost environmental variables. We also aimed to identify potential management targets to protect against Bd infection within our study system. To this end, we developed a Bayesian model to predict swab-based detection of Bd on an individual amphibian using only environmental variables. Our results highlight both the utility and difficulty of this process, as well as identify potential new hypotheses for what controls the occurrence of Bd across a seemingly homogenous landscape. While the data presented here is limited to the coastal Californian vernal system, our findings are likely generalizable to other systems with a strong wet-to-dry seasonality and similar land use.

Materials and Methods

Field Surveys

Twenty sites in the San Francisco East Bay region of California were selected from a larger set of 170 sites whose amphibian populations have been studied extensively since the early 2000's (especially see [30,31,39]). Sites were selected within this larger group based on ease of access and overall representativeness of other sites in the system. Each site consists of a pond or small lake, and sites were selected evenly within property-based blocks (four parks, each containing five sites). Fifteen sites across three blocks occur in public parks while the remaining five are on an ecological preserve. Sites within the public parks are freely accessed by the public and their animal companions, including dogs and horses, as

well as free-ranging cattle. Sites vary greatly in their size, from approximately 300 meters to 20 meters in diameter at their fullest. A unique characteristic of these sites, which is common to many freshwater Californian systems, is the wet/dry seasonal pattern coupled with high rates of ephemerality. Most rain events in the system occur between November and March, and last only a very short period. This region experienced extreme drought conditions through 2012-2015, and drought continues to date [31,32]. Many sites are artificially deepened to reduce their drying while others (up to nearly half of the sites in the driest months) dry completely each summer. Sites also vary greatly in their communities, including presence or absence of turtles, snakes, and amphibian species. Seven amphibian species occur across these sites: *Rana catesbeiana* (American bullfrog), *Pseudacris regilla* (Pacific chorus frog), *Anaxyrus boreas halophilus* (California toad), *Taricha torosa* and *granulosa* (California newt and the granular newt, respectively), as well as two endangered species, *Ambystoma californiense* (California tiger salamander) and *Rana draytonii* (red-legged frogs).

We sampled these sites repeatedly from March 2020 to June 2021, approximately every month during the summer and every other month during the rest of the year, in order to collect information on Bd prevalence in amphibians as well as environmental variables. All equipment including dipnets and boots were disinfected by scrubbing in quaternary ammonium compound 128 (diluted to 0.008% or a higher percentage [33]) between each site visit.

Amphibian Swabs

While all amphibian species and life stages were sampled (except for the endangered *A. californiense* and *R. draytonii*), for analysis we only investigate data from *P. regilla*. This species was chosen because it is common and widespread throughout the year and all sites, and a previous study found that it may drive Bd infection dynamics in this area [39]. Animals were captured using a fresh pair of nitrile gloves or pond-rinsed dipnet. Prior to swabbing, the snout-to-vent length of each individual was measured using calipers. The sex (if possible), species, life stage, and weight is also collected for each individual. Each individual was swabbed (MW113, Medical Wire and Equipment Co.) along the skin to pick up skin cells and zoospores 30 times as follows: five times each on each hind foot, inner thigh, and each drink pouch (if anuran, Hyatt et al., 2007). Animals were then released at the point of capture and clean gloves were used to handle each individual. Swabs were stored dry in Eppendorf tubes, placed on ice for transport, and stored at -8°C in the lab until processing. Genomic DNA was extracted from swab samples using PrepMan™ Ultra Sample Preparation Reagent (Life Technologies, Carsbald, CA) [38] and further cleaned using GeneReleaser according to the manufacturer's instruction (BioVentures Inc., Murfreesboro, TN).

Quantification of Bd using real-time PCR

Quantification of Bd DNA was analyzed using quantitative (real-time) PCR, using an ABI7300 Sequence Detection System (Applied Biosystems). SensiFAST Probe Hi-ROX Kit (Meridian Bioscience) was used as master mix for the PCR reaction. Primers and probe have been described in [38]. Five uL of diluted (1:5 in GeneReleaser) DNA was used in total 25 uL

of PCR reaction. Additionally, TaqMan™ Exogenous Internal Positive Control Reagents (Appliedbiosystems by Thermo Fisher Scientific) were included in the PCR reaction to rule out any inhibitor present in the DNA samples. Laboratory cultured Bd zoospores served as standard control and results are reported in zoospore equivalents per swab (ZE).

Environmental Variables

For each sampling event (i.e., each site at each date of sampling), a series of environmental variables was collected: water temperature of the pond (C, taken at the edge of the pond near the surface), season (wet or dry), perimeter of the pond (standardized paces), trophic state, conductivity (uS), total dissolved solids (ppm), salinity (ppt), pH, presence of cattle, average wind speed (meters per second), multi-day precipitation (mm per day), maximum and minimum air temperature (C), and elevation (meters). Water temperature of the pond, season, pond perimeter, trophic state, conductivity, total dissolved solids, and salinity were collected at the time of sampling, while data from the National Centers for Environmental Information (NCEI [68]) were obtained for wind speed, multiday precipitation, and air temperature. Elevation data was obtained through the Global Biodiversity Information Facility (GBIF) API using the R package rgbif [69]. Water temperature, conductivity, pH, total dissolved solids, and salinity were measured using a hand-held water probe. Season was coded as "wet" if collection occurred in the months of January, February, March, April, or May and coded as "dry" for all other months. Trophic state was coded as either oligotrophic (if the pond had no algae or plant life), mesotrophic (if the pond had some algae or plants), or eutrophic (if the pond was nearly completely filled with algae and plants). Cattle were identified as "present" if cows or fresh cow dung was

observed at the site and "absent" otherwise. Pond perimeter was measured in paces (standardized to a single individual) around the edge of the pond. All variables from NCEI were used exactly as downloaded except for multiday precipitation, which was calculated as the multiday precipitation total divided by the number of days included in the total. Values for NCEI variables were matched to sites based on Manhattan distance to the closest available weather station (maximum Manhattan distance = 0.22595°, minimum = 0.08583°, average = 0.16071°). Latitude and longitude for each site was collected using physical maps of the site cross-referenced against Google Earth.

Statistical Methods

A Bayesian model using a negative binomial likelihood was created with the goal of predicting the zoospore equivalent swab load of an individual *Pseudacris regilla* at a particular site. The negative binomial likelihood was selected due to high counts of zeroes in the dataset, and was chosen due to outperformance of models using a Poisson likelihood (based on LOOIC; Vehtari, Gelman, & Gabry, 2017). In order to conform to the support of the negative binomial distribution, zoospore equivalent swab loads were rounded to the nearest whole number. For individuals whose zoospore equivalent swab loads were between 0 and 1 (6/273 individuals), loads were similarly rounded to the nearest whole number. The model was fit using the stan_gamm4 function in the R package rstanarm [71,72]. Default priors from the rstanarm package were used, which consist of multinomial normal distributions with a mean of zero and scaled standard deviation for all predictors including the intercept; an exponential prior with a rate parameter of one for the auxiliary prior (error standard

deviation), and a decomposed covariance matrix with regularization, concentration, shape, and scale parameters all equal to one, indicating independence between the priors [70,71,73].

Variable selection began by fitting a full model using all possible predictors as linear effects except for water temperature which was included as a thin-plate regression spline based on previous studies' findings that temperature has a non-linear relationship with Bd growth in-vitro [74]. Cubic cyclic splines of both maximum and minimum air temperature were tested compared to linear effects but did not improve model fit. Pond ID number was included as a random effect in all candidate models to control for the additional variation across sites. Pond block was not found to be predictive and thus was removed from the model. Variable selection proceeded by successively dropping a single variable from the model (backwards selection) and calculating either k-foldIC or LOOIC as possible and comparing to the previous model for increased goodness-of-fit [70]. K-foldIC and LOOIC, similarly to AIC and BIC, indicate a relatively better model fit when smaller and are calculated as a trade-off between model fit to the data and model complexity. K-foldIC was calculated instead of LOOIC when too many individual data points were deemed influential based on Pareto-k estimates to increase metric robustness. Once a final set of best predictors was identified, interactions between predictors were checked for improvement of model fit until a single best model was identified.

When a final best-fit set of predictors was identified using the above approach, the model was investigated for potential outliers based on Pareto-k estimates [70] and visual analysis of individual predictors. Finally, model fit and performance was assessed by

checking Markoff chain Monte-Carlo (MCMC) trace plots and visualizations of simulations from the posterior predictive distribution. A "pseudo-R^2" using the ordinary least squares formula was calculated to understand the amount of variance in the data described by the model, as no R^2-like metric is available for the negative binomial distribution due to the support of the distribution.

Results

Field Surveys

A total of 1050 amphibians of the species *Pseudacris regilla*, *Anaxyrus boreas halophilus*, *Rana catesbeiana*, and *Taricha* spp. across the 20 sites were tested for Bd. However, due to the highly seasonally-dependent occurrences of other species and life stages, differences among species in Bd susceptibility, as well as high drying rate of ponds in the system combined with many of the variables surveyed being water-dependent, we focused our analyses on the 273 post-metamorphic *P. regilla* sampled when water was available at the pond. *P. regilla* are the most abundant amphibian throughout the year at these sites, and are a good indicator and potential driver of Bd prevalence [39]. Across all sampling events, we found a Bd prevalence of 23% in *P. regilla* (mean infection load of 1,410 zoospore equivalents per swab, with a maximum of 256,000 zoospore equivalents per swab). Environmental variables and Bd prevalence fluctuated both across sites and seasonally (Figure 1).

Modeling Bd Infection using Environmental Variables

The single best model identified included the snout-to-vent length of the frog, season, trophic state of the pond, presence of cattle, longitude, multi-day precipitation, a thin-plate regression spline of the interaction between water temperature of the pond and log-transformed salinity, and a random effect of pond ID (Figure 2, Table 1, Figure 3). While LOOIC to the next-best model was <1, this model represented the simplest model which captured known biologically-relevant processes of water temperature and salinity[74–76]. The model successfully converged (all Rhats = 1) and MCMC mixing was good (Supplementary Figures 1A-1C). Posterior predictive distributions closely matched the model training data (Supplementary Figure 2). One outlier was identified, observation 186, which had an impossibly high snout-to-vent length and so was removed from the model. All other Pareto-k estimates were below 0.7, increasing confidence in our model estimates (Supplementary Figure 3). The calculated "pseudo-R^2" for the best fit model was 0.071, indicating the best model only describes approximately 7% of the variance in the data. However, this is an invalid metric for models with a negative binomial likelihood and this result should be taken lightly.

Predictors varied in their relationships with predicted zoospore loads (Figure 3). Snout-to-vent length of the frog, longitude, and salinity are all negatively correlated with Bd loads, while precipitation is positively correlated. Taken together, this indicates that larger frogs, in saltier, more easterly ponds have the lowest risk of Bd infection and lower predicted zoospore-equivalent swab loads while frogs at sites with higher precipitation have a higher risk of Bd infection and higher predicted zoospore-equivalent swab loads. Furthermore, the

wet season (as compared to the dry season) predicts higher zoospore loads as do oligotrophic ponds compared to mesotrophic or eutrophic ones. The presence of cattle also predicts lower zoospore loads on amphibians as compared to ponds without cattle. Finally, water temperature had a highly non-linear relationship to zoospore loads, where mild temperatures have the highest Bd loads and very cold or very hot water temperatures predict little to no Bd load.

Discussion and Conclusions

In the above work, we have investigated how environmental variables can predict presence and intensity of Bd infection in *Pseudacris regilla* in our study system. Out of a large suite of variables assayed, we found that water temperature, salinity, precipitation, longitude, trophic state of the pond, season, presence of cattle, and size of the individual frog were the best predictors.

Our results on water temperature and salinity echo previous findings that very high or low water temperatures (lower than about 8 degrees C or above about 27 degrees C [74,76–79]) and salinity concentrations of over about 2 ppt [76,79–81] reduce risk of Bd infection and zoospore load. Interestingly, our finding that a bivariate thin-plate regression spline of the two variables outperformed simple linear effects of both, or even a thin-plate regression spline of just water temperature, indicates there is an important interaction between water temperature and pond salinity. The lowest Bd risk and zoospore loads occur at extreme water temperatures and high salinity, indicating that this finding could be exploited to enhance management applications which may only focus on adding salt to the pond. By

waiting to increase pond salinity until very cold or hot times of year the efficacy of the salt treatment may be improved; yet, this would need further testing in a mesocosm setting.

The results on precipitation, longitude, trophic state, season, and snout-to-vent length are likely much less useful for management after Bd arrival at a site, yet may be important indicators for sites that are at high risk. Various studies have found different relationships between precipitation and Bd risk, including findings of an interaction between season and precipitation which we found no evidence of in our system [64,82–86]. This may indicate an interesting, regional change in how precipitation affects Bd occurrence and is a topic ripe for further investigation. In this system no rain events occur outside of a period from November to about March and this study occurred during a drought period, therefore this high seasonal dependence of precipitation may prevent transferability of these findings to systems where precipitation occurs throughout the year or at higher rates. Furthermore, longitude itself is likely not the true predictor but a covariate for some other biological process or simply an artefact of our specific study system. Interactions between longitude and salinity were not strongly predictive, indicating this result is likely not due to seawater encroachment. This result is also not attributable to pond block, as that was not found to be a predictive factor in the model selection process. While little experimental evidence for the effect of pond trophic state on Bd risk exists, two major hypotheses have been brought forward: (1) increased eutrophy will increase diversity of parasites and hosts and therefore increase pathogen risk; (2) increased eutrophy will increase diversity of parasites and hosts and therefore decrease pathogen risk [87]. We found a weak relationship of trophic state and Bd risk in our system, however we found that an oligotrophic pond was a predictor of increased Bd risk as

compared to mesotrophic or eutrophic ponds, which may lend support to the latter hypothesis, however more evidence will need to be collected to support this claim. Our finding that the wet season carries a higher risk of Bd infection in comparison to the dry season is intuitive for our system: amphibians flock to the pond in extremely high numbers during the wet season to breed, and nearly all adults move away from the pond by the dry season, leaving only post-metamorphic yearlings. Therefore, it may be that it is not the season itself, but the breeding event, which may be driving this relationship. Given that Bd is a pathogen with density-dependent transmission [88,89], a dramatic increase in population size during the wet season may drive an increase in transmission and therefore Bd risk and load. Finally, our finding that smaller-bodied *P. regilla* are more at-risk of Bd infection than larger individuals indicates that size does matter in terms of infection, and should be included in other predictive models in order to ensure accurate predictions. This finding of a negative relationship between body size and Bd risk is not unique to our system [84,90,91], yet species and age of the individual may be an interacting factor in how size of the amphibian predicts Bd risk [92,93].

One of our most striking findings was the highly predictive relationship between the presence of cattle and decreased Bd risk. This is in contrast to a previous finding of no effect of grazing on Bd occurrence in this study system [30]. While it is often hypothesized that livestock increase Bd risk to amphibians, little research exists on this topic (but see [94]) and we found no evidence to support this claim. In fact, ponds where cattle were either actively or very recently present (as determined by presence of fresh dung) had lower Bd presence and loads. One potential mechanism for this relationship could include cattle waste altering

the microbial composition of the pond environment to decrease the viability of Bd, and would be an interesting target for future work and even potential management actions. However, this work should be carefully replicated in many other systems to ensure this effect is not unique to our study system, especially because the effects of cattle on amphibians remains a debated topic and is likely system- and species-specific [95].

Despite the identification of strongly predictive variables for Bd risk and zoospore load, we have low confidence in this model to predict zoospore loads on *P. regilla* even within the study system. While the simulated posterior predictions fall neatly along our observed data, indicating the model predicts the data fairly well, there is an unusual oscillating trend present in some simulations from the posterior distributions which is not found in our data (Supplementary Figure 2). When performing model selection, LOOIC decreased only a few points between the full and best model, indicating that there is not a great difference between the models. Finally, while not truly a valid metric for understanding how much variance in our data is captured by the model, the pseudo-R^2 of only 7% indicates, at least, that we are capturing only a very small piece of the picture of what drives Bd occurrence and intensity across a relatively homogenous landscape. It is likely that other factors, including host community composition and transmission dynamics, are playing a larger role than environmental variables in this system. Therefore, while the findings of this model may be highly useful for specific and targeted management actions, we do not recommend using this model to generate specific Bd risk and load predictions.

Taken together, our results confirm previous work that extreme water temperatures and high salinity may exert a protective effect against Bd. Furthermore, we have identified new possible targets for management actions, including eutrophication and addition of cattle, yet we present these targets with a high degree of caution. Experimental studies should be performed in a wide variety of systems in order to confirm these relationships before attempting to alter lentic ecosystems in such drastic ways. Finally, we found that while we can identify predictive variables for Bd risk and load, we cannot confidently predict Bd risk and load for a frog under given conditions, indicating that other host-pathogen processes may be playing a larger role than the environment in our system.

Figures and Tables

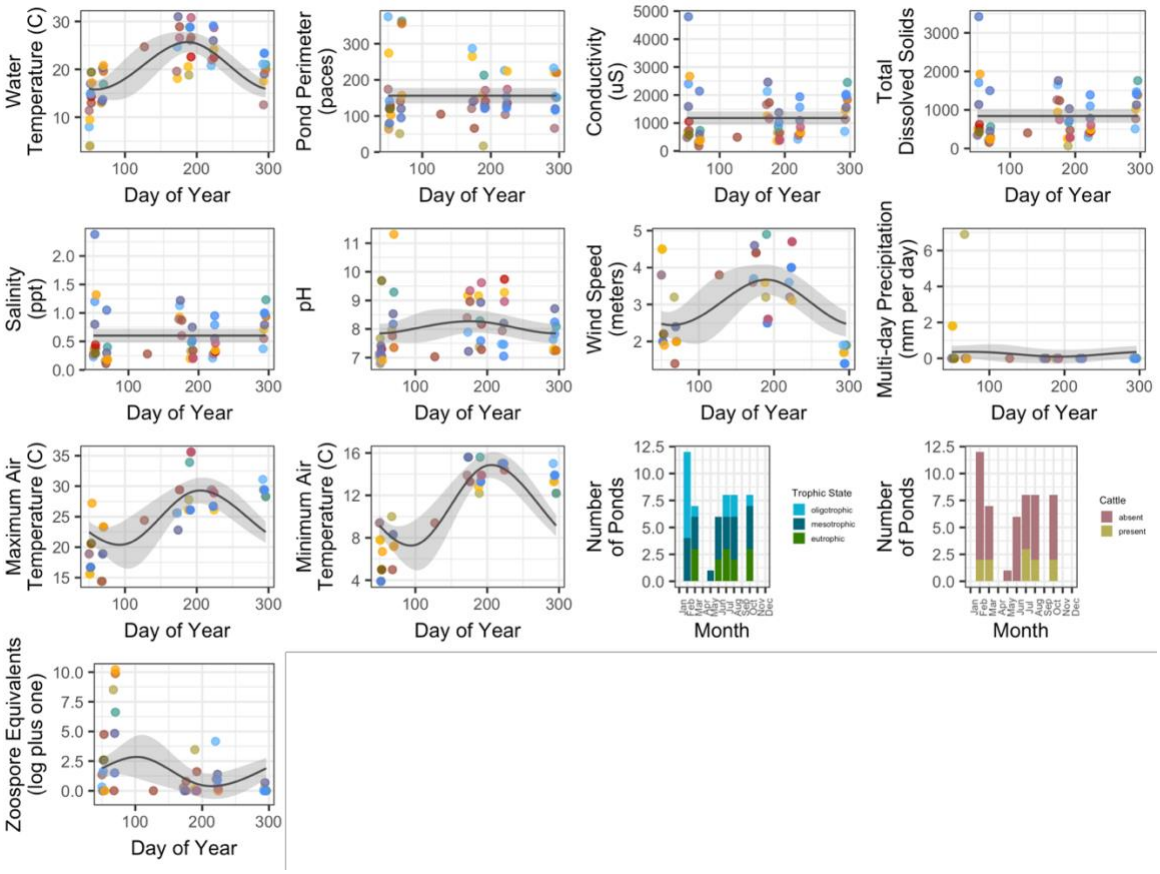


Figure 1. Each plot shows the environmental variables (and zoospore equivalents per swab) throughout the year. Different colored points indicate different ponds. Black lines show cyclic cubic smooths with a basis dimension of 4 and 95% confidence interval ribbons.

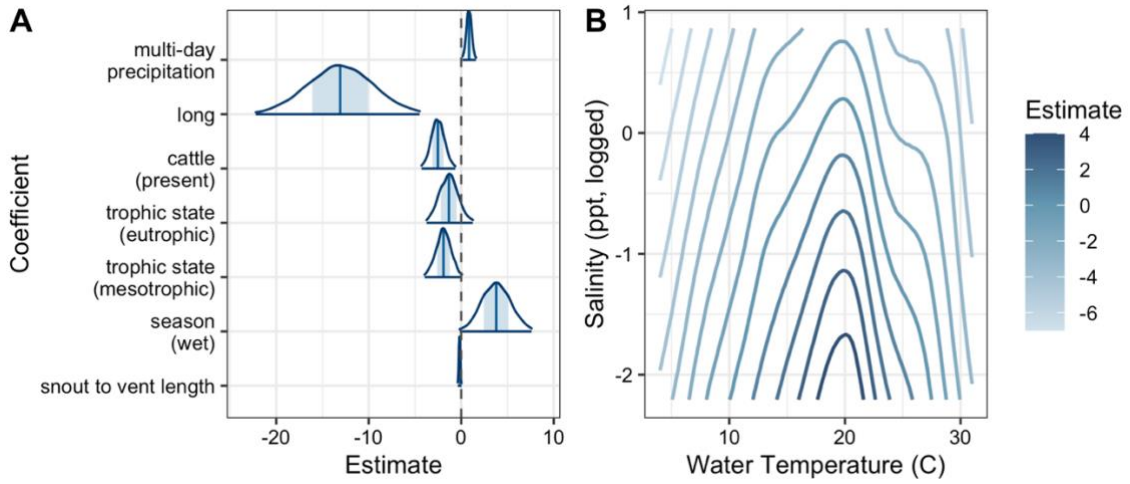


Figure 2. (A) shows the central posterior interval estimates from MCMC draws for the parameter indicating the effect of each variable. Positive values indicate an increase of Bd load while negative values indicate a decrease in Bd load. Shaded blue areas show the 50% credible interval, the entire density represents the 95% credible interval, and the medians are given as a blue line. (B) shows the central posterior interval estimates for the bivariate (interaction) thin-plate regression spline between water temperature and logged salinity at a credible interval of 90%.

Table 1: Estimates for the linear predictors in the model.

	Estimate	Standard Error
intercept	-1589.31	541.10
snout to vent length	-0.17	0.11
season (wet)	3.79	1.97
trophic state (mesotrophic)	-1.92	1.01
trophic state (eutrophic)	-1.33	1.27
cattle (present)	-2.52	0.90
longitude	-13.08	4.45
multi-day precipitation	0.85	0.36

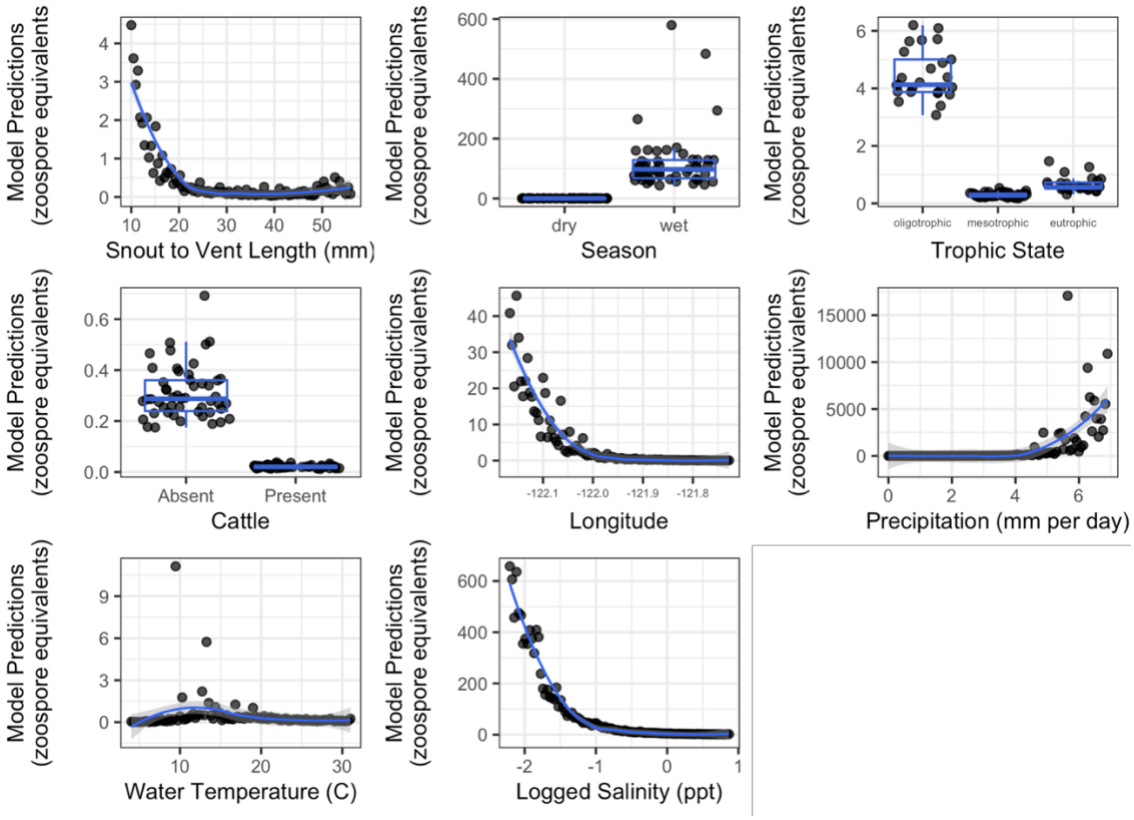


Figure 3. Predictors were fixed at medians (or, for categorical variables, at the most frequent level) except for the predictor indicated in the graph, which were varied to contain the full range of observed values over 100 data points. The maximum number of posterior predictions (n simulations = 7,960) was then generated using this new dataset in order to visualize how the value of the predictor affects model predictions. Posterior predictions were averaged for each value of the predictor and plotted (n averages = 100). Blue lines indicate loess smooths and ribbons represent 95% confidence intervals for numeric variables, or boxplots for categorical variables.

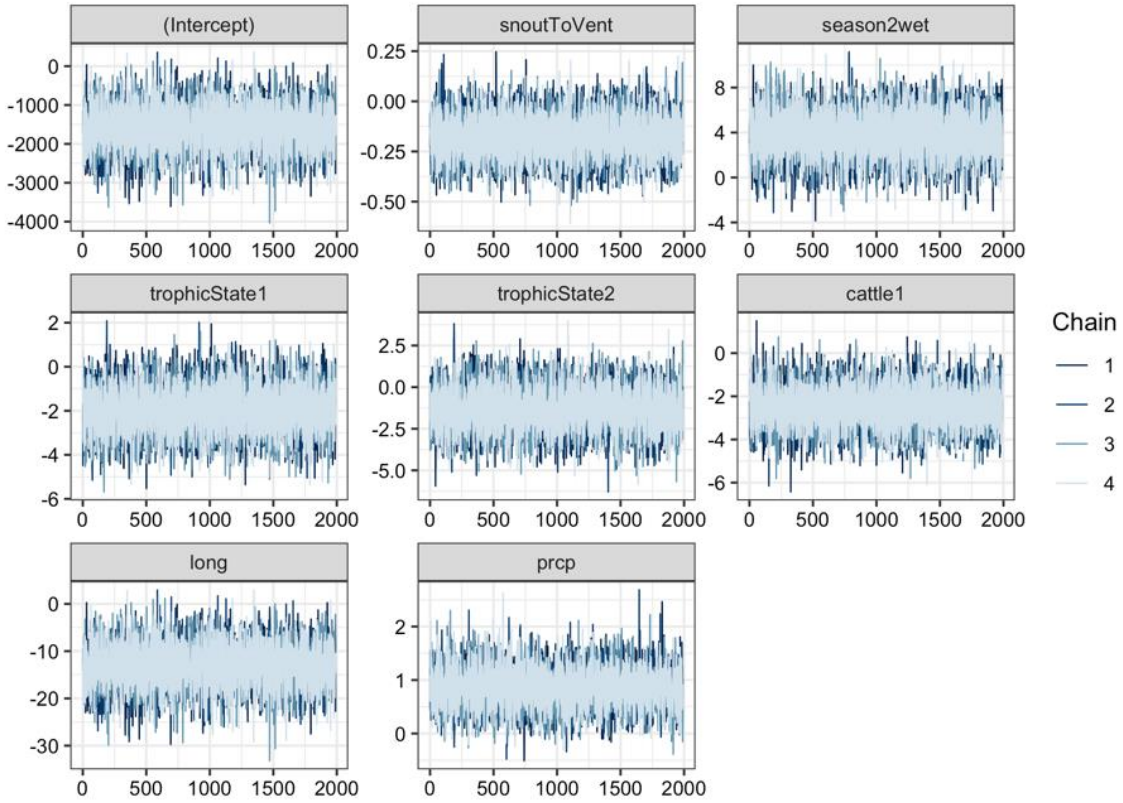
Acknowledgements

We thank the NIH, Worster Family, and Schmidt Family Foundation for funding. We thank the undergraduate student researchers without whom this work would not be possible: Alyssa Byer, Yanelyn Perez, Kana Suzuki, Sophie Boshoff, Sarah Boyle, Kaylie Nguyen, Grace Kloss, Hope Hahn, Kathryn Koo, and Katie Hampton. We thank the University of Colorado at Boulder field team under Dr. Pieter Johnson, especially Dr. Travis McDevitt-Galles, Dr. Wynne Moss, and Brendan Hobart.

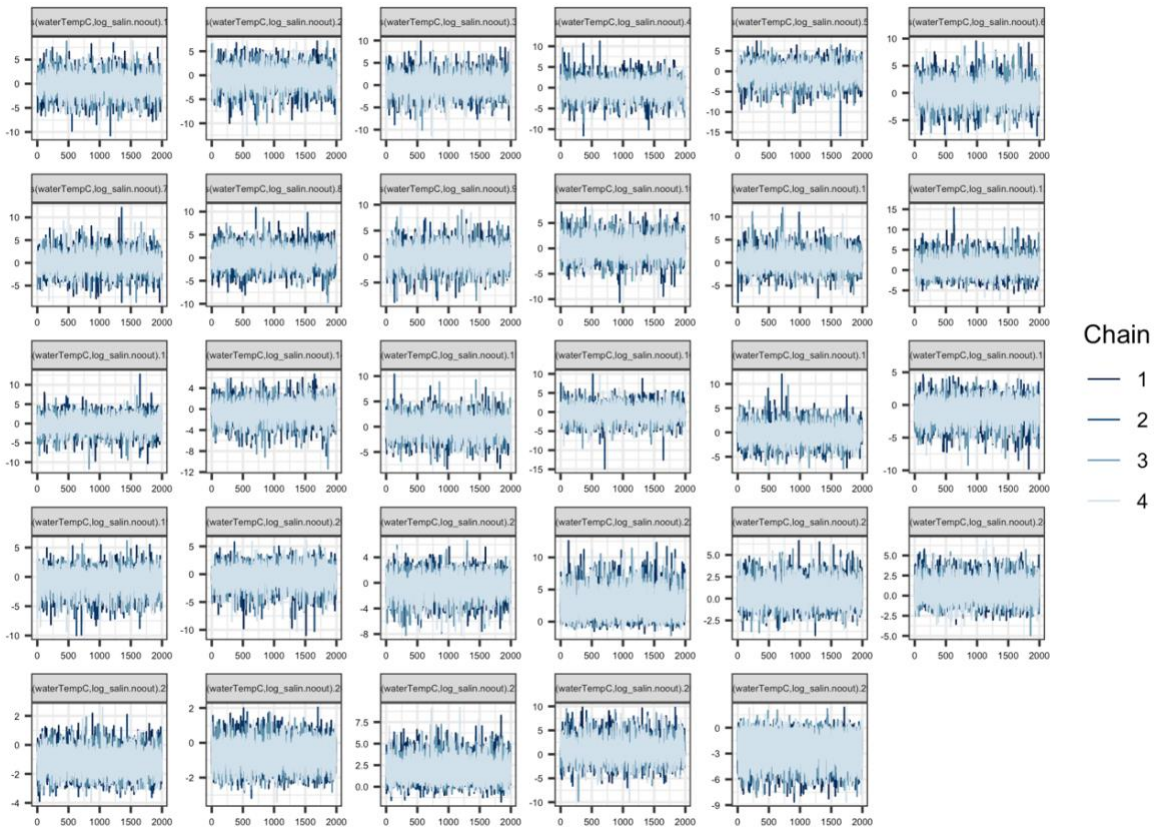
Ethics Statement

This study and its methods were approved by the University of California Santa Barbara's Animal Use and Care Committees (protocol No. 735.3). Permission to collect *P. regilla*, *B. boreas*, *R. catesbeiana*, and *Taricha spp*. was granted by the California Department of Fish and Wildlife (permit Nos. SC-010167 and S-190450003-20052-002). Permission to perform research at specific field sites was granted by the East Bay Regional Park District (research permit No. 19-1018) and the University of California Natural Reserve System (application No. 40947).

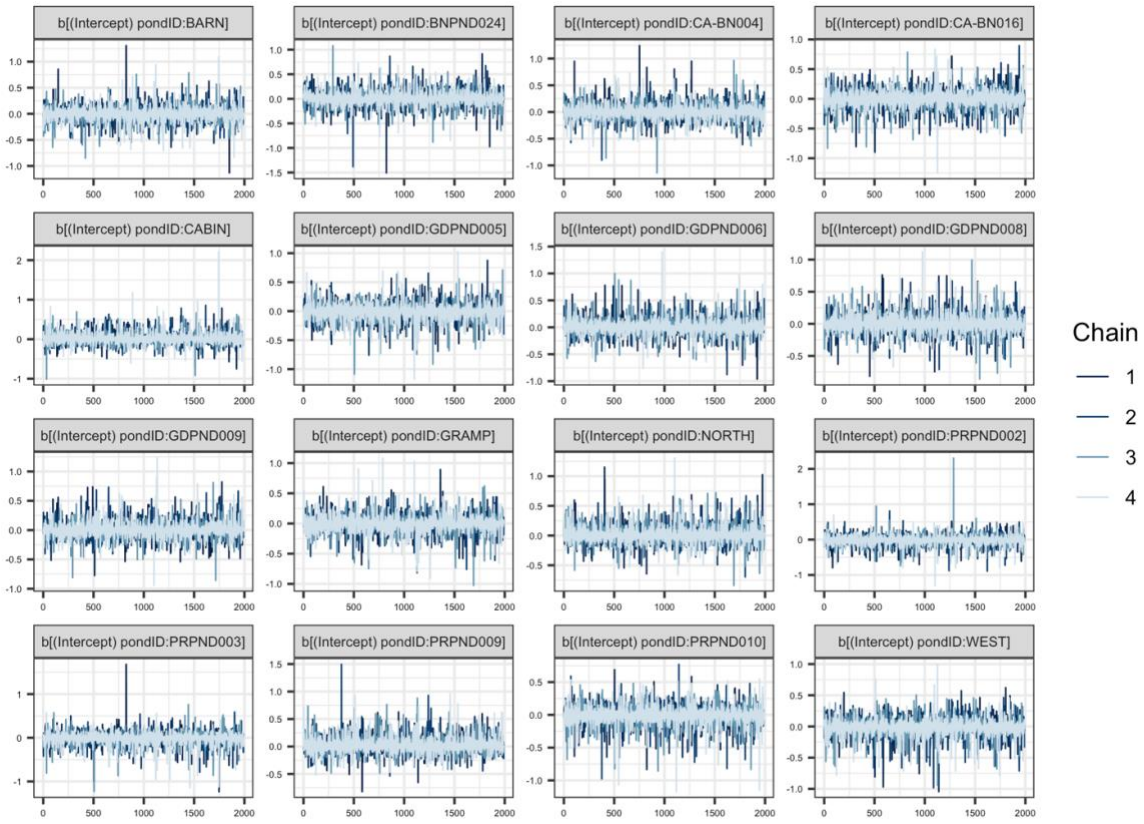
Supplementary Materials



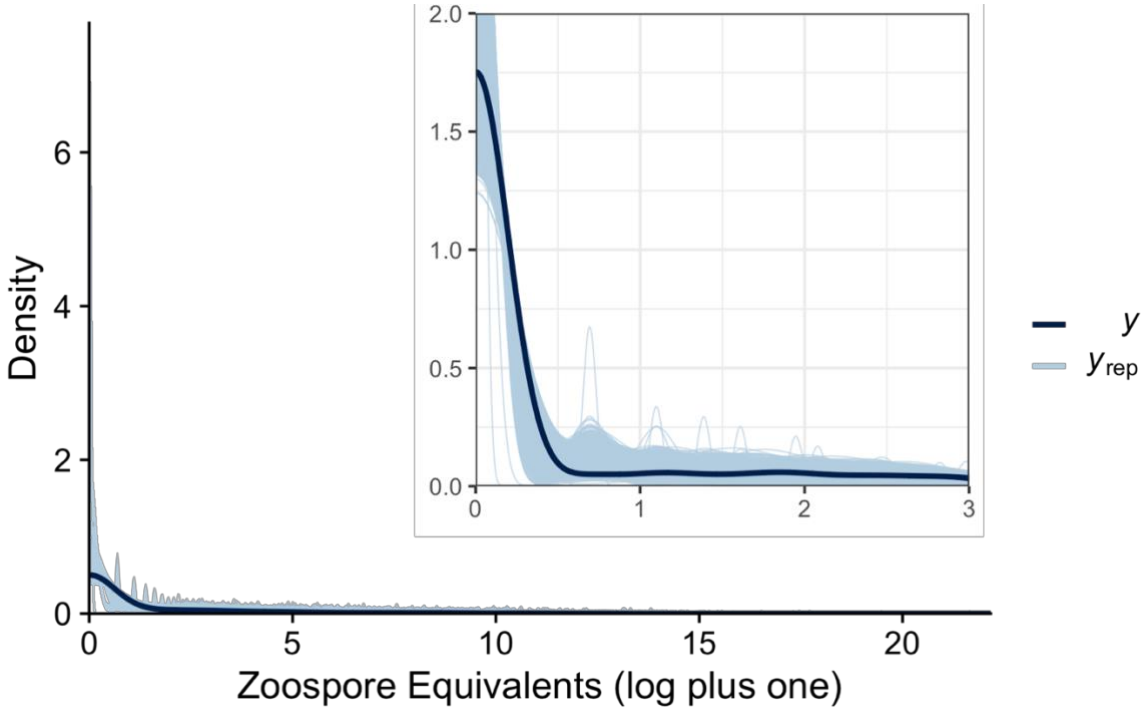
Supplementary Figure 1A: MCMC trace plots for the Intercept and Linear Effects. Plots show even mixing of chains throughout sampling and lack of autocorrelation, indicating good MCMC sampling.



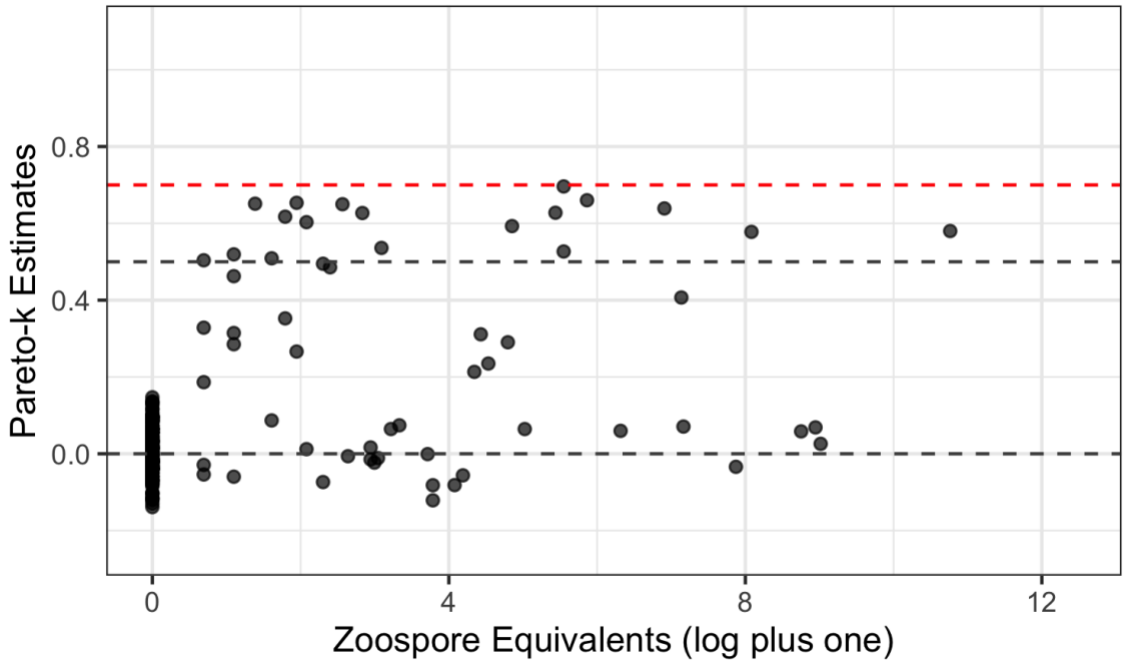
Supplementary Figure 1B: MCMC Trace Plots for the Bivariate Thin Plate Regression Spline. Plots show even mixing of chains throughout sampling and lack of autocorrelation, indicating good MCMC sampling.



Supplementary Figure 1C: MCMC Trace Plots for the Random Effects. Plots show even mixing of chains throughout sampling and lack of autocorrelation, indicating good MCMC sampling.



Supplementary Figure 2: Posterior Prediction Checks. The maximum number of posterior predictions (simulations from the model) were simulated (n=7,960) and plotted (light blue, Yrep) beneath the actual data (dark blue, Y) on the log scale for ease of viewing. This plot indicates a generally good fit of the model predictions (Yrep) in most simulations to the data (Y), with some simulations developing an oscillatory pattern indicating poor fit for combinations of certain predictor values.



Supplementary Figure 3: Pareto-k Estimates. Pareto-k estimates for each observation were calculated for use as a metric of influence and plotted against the zoospore equivalent swab loads for that observation (Vehtari 2017). Gray dashed lines indicate estimates of 0 and 0.5, while the red dashed line indicates a value of 0.7, above which points are considered influential.

Chapter 3

Drosophila melanogaster is a potential vector of Batrachochytrium dendrobatidis

Authors

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Abstract

The fungal pathogen *Batrachochytrium dendrobatidis* (Bd) causes the disease chytridiomycosis in amphibians and is responsible for mass die-off events and species declines in many amphibian populations all over the world. Previous observational studies have shown that Bd may be vectored by nematodes, waterfowl, lizards, and crayfish; therefore, we sought to determine the vector capabilities of invertebrates in a laboratory setting. We use the insect *Drosophila melanogaster* as a model organism to determine if insects could possibly be a vector for Bd. Our findings show that *D. melanogaster* can test positive for Bd for up to five days post-exposure and can transmit Bd to conspecifics without suffering mortality. Insects of all types interact with the amphibian habitat and

amphibians themselves, making this a potentially important route of transmission between both amphibians and sites in the wild.

Introduction

The emerging infectious disease Chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), has led to global amphibian population declines and species extirpations [8,54,56]. Bd infects the keratinized skin of an amphibian host, disrupting the flow of electrolytes across the membrane ultimately leading to cardiac arrest [96]. While much of the research on Bd has focused on its effects on amphibian populations, increasing evidence suggests that Bd may be a generalist fungal pathogen with non-amphibian hosts and reservoirs. An experimental study detected Bd in sterile pond substrate and bird feathers [36], while others have detected Bd DNA on lizards [97], birds [98], leaves [99], nematodes [34], crayfish [100,101], and have successfully infected zebrafish in a laboratory setting [102]. Yet, many of these studies' findings have yet to be replicated, and furthermore, to-date the Bd detection on potential vectors in natural systems is solely DNA based. DNA is problematic as a means of identifying potential reservoirs and vectors in the wild as detected DNA may be from nonviable or noninfectious zoospores. DNA may last in the environment for hours to days following the death of the host organism[50].

Hosts and reservoirs can help maintain a pathogen in the environment, increasing prevalence and allowing it to drive the focal host extinct [6]. Traditional disease theory predicts that a pathogen with density-dependent transmission, such as Bd, would be eliminated following a mass mortality of its host because there are too few individuals to

maintain it in the population - this is known as epidemic fade-out [6,88]. However, Bd has not experienced epidemic fade-out. If an environmental reservoir exists, it would explain how Bd continues to spread despite extirpation of its hosts. Furthermore, Bd is a member of the phyla Chytridomycota [20]. Most other chytrids are free-living detritivores or invertebrate parasites, with Bd and its sister species (which infects salamanders, *Batrachochytrium salamandrivorans)* being unique in their ability to infect vertebrates [23].

While observational evidence builds that Bd may have non-amphibian hosts and reservoirs, few laboratory studies have been done to quantify and parameterize these interactions (but see the work done on nematodes, i.e. [34,35]). Quantifying and understanding the importance of these non-amphibian hosts and vectors is key to our ability to control and manage Bd-related amphibian population declines. Furthermore, laboratory studies which detect Bd through DNA have an advantage over their observational counterparts in that the only possible source of Bd DNA is through viable spore rather than contact with spore debris in the natural environment. Therefore, we sought to determine if *Drosophila melanogaster* could be inoculated with and transmit Bd in a laboratory setting. *D. melanogaster* has been used as a model organism since the early 20th century [103] and is very commonly used as a feeder animal for captive amphibian colonies. We selected it due to these qualities and to represent other short-lived, winged insects which may interact with multiple, otherwise isolated, amphibian habitats. Here, we present a series of experiments and methods which demonstrate that *D. melanogaster* can be inoculated with and maintain Bd as well as transmit Bd to conspecifics, without suffering any mortality by the pathogen.

We also estimate the transmission function for *D. melanogaster* to *D. melanogaster* transmission in order to the quantify the interactions between this potential vector and Bd.

Materials and Methods

Bd husbandry

Bd was cultured on 1% tryptone agar plates one week prior to each experiment. Inoculum consisted of a cocktail of four Bd strains isolated from the Sierra Nevada: TST77, CJB4, CJB5-(2), and CJB7. Concentrations of Bd were estimated by hemocytometer, while Bd zoospore viability was visually identified via microscopy.

Animal husbandry

Nonsterile *Drosophila melanogaster* were maintained in plastic containers with homemade fruit fly media along with excelsior. Preliminary experiments showed that pre-mixed fruit fly media, which contains antifungal substances, prevented Bd growth and inhibited infection of *D. melanogaster*. Therefore we used a homemade media that relied on dilute vinegar as an antimicrobial and which does not inhibit Bd dynamics. Flies were transferred to new containers approximately every two weeks.

Inoculation Experiment

To determine if *D. melanogaster* could be inoculated with and maintain Bd, we inoculated flies and observed them for five days. For inoculation, 1% tryptone agar plates

with live Bd (10^6 Bd zoospores per fly), heat-killed Bd (10^6 Bd zoospores per fly), or water were created with ten flies per plate. Bd inoculum was spread across the agar plate and allowed to thoroughly dry down before flies were added to the petri dish. Flies were able to move around inside the petri dish and were incubated with the Bd for 24 hours. The treatment of heat-killed Bd was included to see if different fungal loads were detected between live and dead Bd. We created one agar plate per treatment for each of five days (n = 10 flies per day, sex, and treatment combination, Supplementary Table 1). Flies were separated based on sex because it has been shown that flies have X-linked variation in immune response, which may result in differing fungal load [104]. This experiment was replicated three times: two trials were conducted containing all three treatments, while one trial contained only the treatments of live Bd and water (as a negative control, Supplementary Table 1). After 24 hours, flies were transferred to vials with homemade fruit fly media. Flies were euthanized at -4° C each day for five days, and half of each group of ten flies was washed with 500µL of PBS buffer per fly to remove external, passively-adhered Bd or Bd DNA. Bd load was evaluated with qPCR, with five flies per sample. To test if treatment, day, or sex predicted Bd load, a linear mixed-effects model was developed with fly vial as a random effect using the R package lme4 [41].

Dosage Dependency

A dosage dependency assay was conducted to discern the optimal concentration of Bd to inoculate fruit flies. Groups of eight flies of each sex were placed on 1% tryptone agar plates containing the following concentrations of Bd zoospores per fly: 0, 10^3, 10^4, 10^5, and 10^6. Flies were left on the plates for 24 hours, then transferred to fly vials and left for an

additional 24 hours. Flies were then euthanized at -4° C. Half of the flies were washed with PBS as above prior to DNA extraction to remove external Bd to try to discern if Bd is present on the exoskeleton or internally (n = 8 flies per sex, washing treatment, and dosage treatment combined, Supplementary Table 2). Bd load was evaluated with quantitative PCR, with five flies per sample. To test if dosage, washing, or sex treatments produced significantly different mean Bd loads, a three-way ANOVA was run followed by a Tukey-Kramer test to identify the significantly different groups [105].

Mortality Assay

To see if Bd has negative effects on *D. melanogaster*, a mortality assay was conducted. Five flies each were incubated on eight, 1% tryptone agar plates with each of three treatments: 10^6 Bd zoospores per fly, Bd supernatant, and water. Bd supernatant was included because evidence suggests that Bd supernatant interferes with cell junctions and immune cell function in amphibians [106]. Flies were again split by sex (n = 20 flies per treatment, per sex, Supplementary Table 3). Flies were left on the plates for 24 hours, then transferred to fly vials containing homemade fruit fly media. Fly deaths were recorded daily until the end of the experiment (day 5). To determine if treatment groups had significantly different mortality, Kaplan-Meier survival curves were fit to the data and a Log-Rank test comparing the curves was run using the R package survival [107].

Transmission Experiment

To see if *D. melanogaster* could transmit Bd to conspecifics, a transmission experiment was conducted. All flies were inoculated in groups of 10 per agar plate, dosed with 10^6 zoospores per fly. We followed the treatment scheme density and initial infection prevalence of Tompros et al. [108]. Flies were incubated on inoculation plates for 24 hours, then transferred to empty fly vials according to treatment plan. Flies were euthanized by freezing 24 hours later, and moved into individual reaction tubes (one fly per reaction) for DNA extraction. We followed the approach of Rachowicz and Briggs [88] to determine the best-fit transmission function using a maximum likelihood approach and AICc in MATLAB [109–111].

Molecular Analyses

Whole *D. melanogaster* were digested with Proteinase K following the manufacturer's directions and DNA was subsequently purified using the DNeasy Blood & Tissue Kit (QIAGEN) following manufacturer's directions. Quantification of Bd DNA was analyzed with an ABI7300 Sequence Detection System (Applied Biosystems). SensiFAST Probe Hi-ROX Kit (Meridian Bioscience) was used as master mix for the PCR reaction. Primers and probe have been described in [38]. Five uL of DNA was used in total 25 uL of PCR reaction. Additionally, TaqMan™ Exogenous Internal Positive Control Reagents (Appliedbiosystems by Thermo Fisher Scientific) was included in the PCR reaction to rule out any inhibitor present in the DNA samples. Inhibited samples were washed a second time in spin columns following manufacturer's directions. Laboratory cultured Bd zoospores served as standard control and results are reported in zoospore equivalents (ZE).

Results

Inoculation Experiment

All *D. melanogaster* in both the heat-killed and Bd-positive treatments had positive qPCR results for Bd, with no significant difference in load between the treatments (Welch's t = -1.413, p=0.1624, alpha=0.05). No individuals in the negative control treatment had Bd positive qPCR results. Furthermore, days post exposure and sex had no effect on Bd load ($p > 0.05$), while washed flies had significantly lower loads ($t = 6.535$, $p = -5.9 \times 10^{9}$, alpha=0.05, Figure 1).

Dosage Dependency

All flies in all treatment groups, except those dosed with 1000 zoospore per fly, were Bd positive; within the 1000 zoospore dose group, 50% of the flies were Bd positive. No flies from the negative control group tested positive for Bd. Inoculation dose was a significant predictor of Bd load ($f = 48.531$, $p = -2.27 \times 10^{13}$, alpha=0.05), while sex and washing treatment were not significant predictors ($p > 0.05$, Figure 2).

Mortality Assay

We found no significant difference in survival curves across any of the treatment groups ($\chi^2 = 18.6$, p-value = 0.70, alpha = 0.05), indicating there was no effect of sex, fly vial, or Bd treatment in determining fly mortality (Figure 3).

Transmission Experiment

During creation and analysis of the experimental treatment groups, the n=20 groups for both 12.5% and 50% infection prevalence were lost. Furthermore, rather than an n=32, 25% prevalence group, an n=34, 24% prevalence group was created. Data were fit to various transmission functions: constant risk, density dependent, frequency dependent, power, asymptotic, and negative binomial as discussed in [88,112]. Estimates for function parameters and AICc values are given in Table 1. The function with the lowest AICc value was the power function (AICc = 62.51), and ΔAICc to the next-best function (density dependence) was 1.19, indicating little to no discrimination between these two transmission types. However, there was moderate discrimination between the best function (power) and all other functions (ΔAICc > 2).

Discussion and Conclusions

Taken together, the results of our experiments suggest that not only can *D. melanogaster* be inoculated with Bd at ecologically-relevant loads and not suffer mortality, but can even transmit Bd to conspecifics. No differences were found between the sexes, suggesting *D. melanogaster* sexes do not differ in their immune response (if any) to Bd. In the inoculation and dosage-dependency experiments, flies were Bd positive even after thorough washing, indicating that the positive result is likely not due to passively adhered Bd DNA, but instead viable Bd zoospores that may be adhered internally or externally to the fly.

Washing the flies in PBS solution only significantly reduced Bd loads in the inoculation experiment, indicating DNA detected from Bd is more than passively attached to the flies.

However, as flies in the inoculation experiment dosed with heat-killed Bd and subsequently washed still returned positive Bd qPCR results, this indicates that even dead Bd has the ability to stay attached to an organism. This provides evidence that detecting Bd DNA through PCR methods is not inherently indicative of viable Bd, much less Bd infection. Many other studies have successfully detected Bd on various substrates in the amphibian habitat [53,97,98,101,113], yet these may not indicate true vectors or reservoirs of Bd based on our findings. This result is further supported by the lack of a significant difference between Bd qPCR results of live vs. heat-killed Bd in the inoculation experiment.

In the dosage-dependency experiment, we found that flies could be inoculated with Bd at doses as low as 1000 zoospores per fly and we obtained 100% Bd positive results at 10,000 zoospores per fly. In natural settings during an outbreak, a single frog may produce a swab with tens of thousands of zoospores [114,115] and a milliliter of water can have about 1000 zoospores [47]. Flies in natural settings come into contact with water and amphibians to gain moisture, representing a possible transmission pathway between not only bodies of water in a system, but between amphibians themselves. Unexpectedly, exposure to both Bd and Bd supernatant had no effect on fruit fly mortality, indicating that if the flies are truly experiencing a genuine infection, they are able to tolerate it well. Whether the flies are truly infected by Bd or simply acting as a vehicle of transmission is beyond the scope of this work, yet our findings demonstrate that *D. melanogaster* certainly has the potential to be a vector for Bd in natural settings.

While our transmission experiment could not discriminate between a power function or a density-dependent function for transmission, we nonetheless observed transmission between *D. melanogaster* in the laboratory setting. This aligns with findings from other amphibian disease studies; Rachowicz and Briggs [88] found that Bd transmission in the wild was best described by a density-dependent function, while Greer and colleagues [112] could not discriminate between a power function and a negative binomial function for *Ambystoma tigrinum* virus (ATV). Interestingly however, Tompros and colleagues [108] found the best support for frequency-dependent transmission for Bd's congener *Batrachochytrium salamandrivorans* (Bsal). While few studies exist which attempt to quantify transmission functions for amphibian diseases, the variety in results here suggests that transmission is context dependent between host and pathogen, while the inability to confidently discriminate between multiple transmission functions outlines the difficulty in estimation of transmission functions across these systems. Despite lack of clarity on transmission function, we have shown that *D. melanogaster* can transmit Bd to conspecifics which further contributes to their ability to be a vector in the natural environment.

Unfortunately, our study is severely limited in its conclusions in that it consists of laboratory experiments with artificially inoculated insects and relies solely on DNA-based Bd detection. Future work should explore the relevance of our findings in the natural setting, and further explore whether infectious Bd can persist on *D. melanogaster*. Finally, laboratory experiments to determine of *D. melanogaster* can transmit Bd to amphibians would be a critical next step. Here, we have presented work and validated methodology for

use in a lab setting to help answer these pressing questions, and hope our work can be used as a framework for validating other non-amphibian hosts of Bd.

Figures and Tables

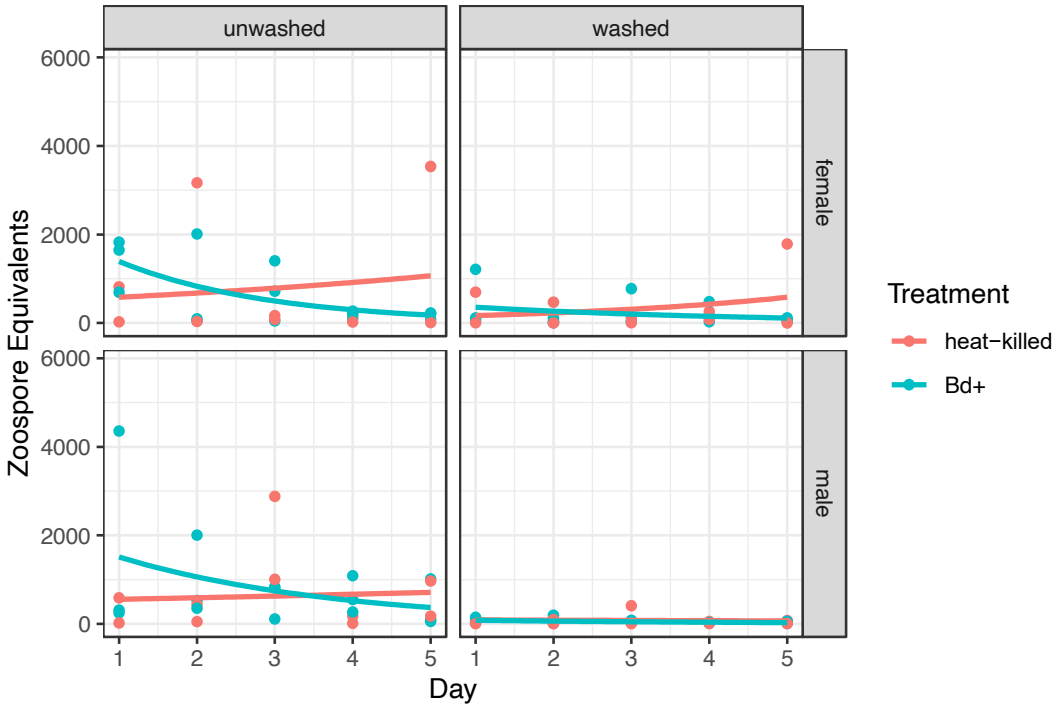

Figure 1. *D. melanogaster* were inoculated with Bd or heat-killed Bd and sacrificed each day for five days. Fit lines follow a Poisson distribution and shaded ribbons represent 95% confidence intervals.

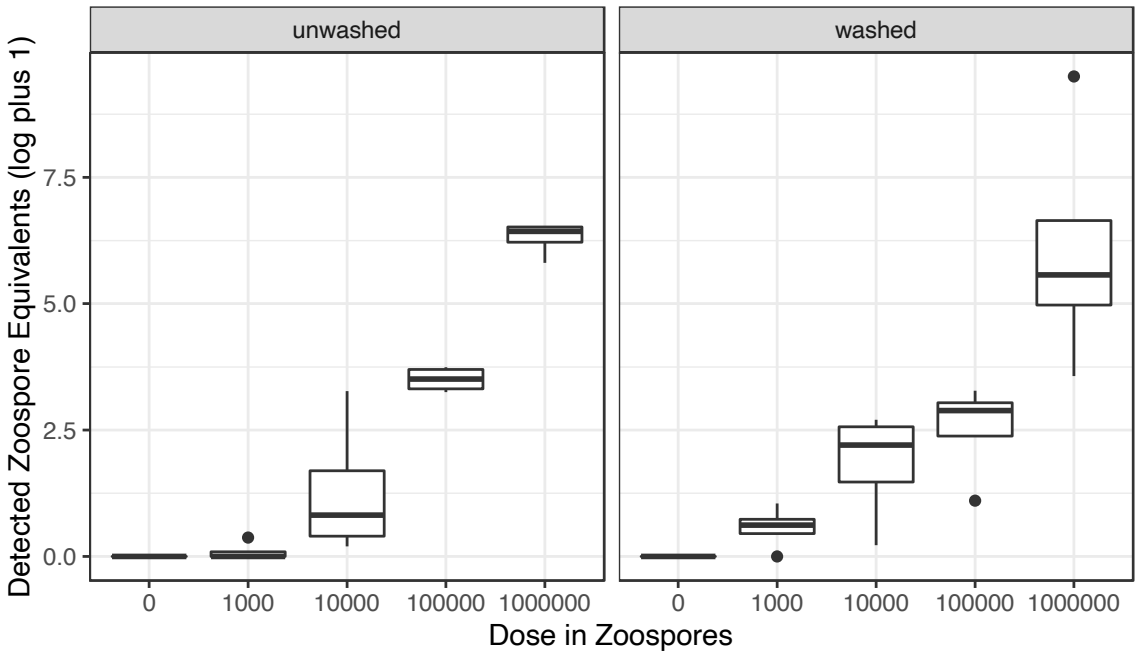

Figure 2. *D. melanogaster* were inoculated with Bd at a range of concentrations. There was no significant difference between sexes therefore results are pooled across sex.

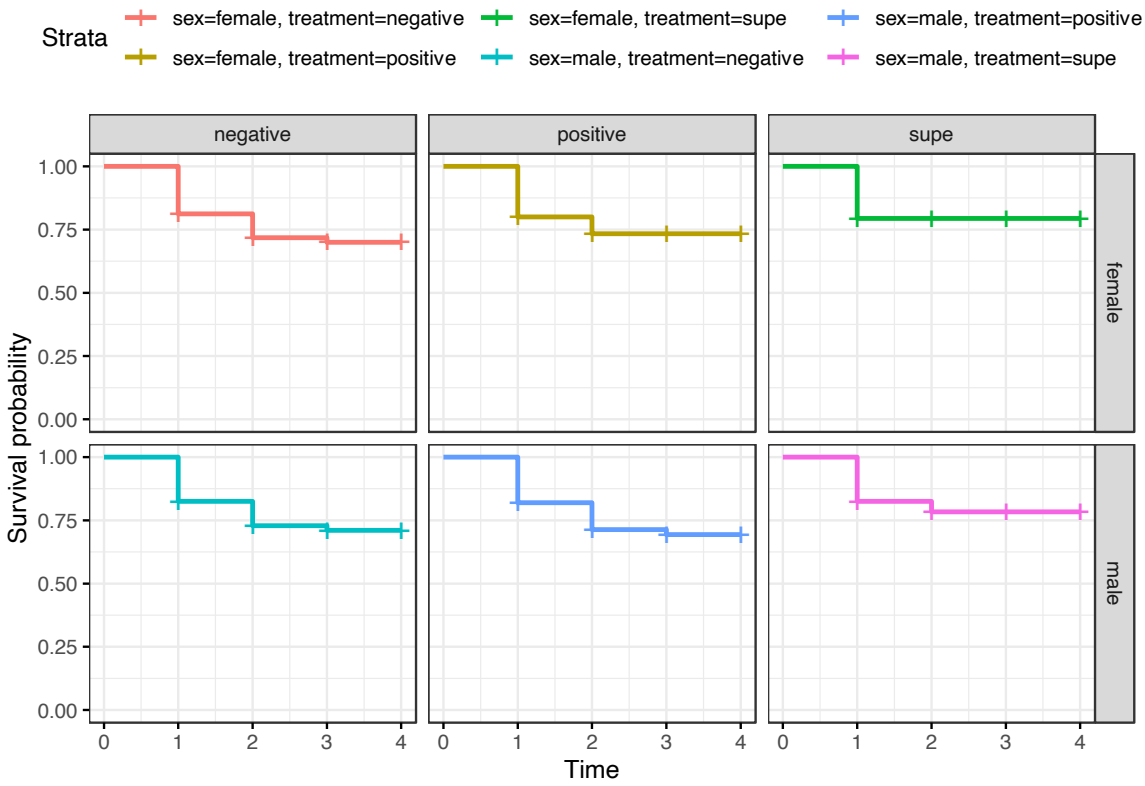

Figure 3. Flies were split by sex inoculated with either live Bd spore, Bd supernatant, or water (as a negative control) and monitored for five days for mortality.

Table 1: Transmission Function Results.

Transmission Type	Function	Parameter Estimates	AICc	ΔAICc
Power	βSI^q	$\beta = 0.0012$ $q = 2.16$	62.51	0.00
Density Dependent	βSI	$\beta = 0.015$	63.70	1.19
Asymptotic	$\frac{\beta SI}{(c+S+I)}$	$\beta = 0.63$ $c = 18.51$	64.90	3.39
Negative Binomial	$k \ln\left(1+\frac{\beta I}{k}\right) S$	$\beta = 8.79 \times 10^{-5}$ $k = 170.99$	66.09	3.58
Constant Risk	βS	$\beta = 0.097$	75.93	13.42
Frequency Dependent	$\beta SI/N$	$\beta = 1.00$	97.29	34.78

Supplementary Materials

Supplementary Table 1: Inoculation experiment. Table rows and columns give treatments, while cell values indicate the number of flies used in that treatment.

Day	Male Control (water)	Male Treatment (10^6 zsps/fly)	Male Heat-Killed (10^6 zsps/fly)	Female Control (water)	Female Treatment (10^6 zsps/fly)	Female Heat-Killed (10^6 zsps/fly)
1	30	30	20	30	30	20
2	30	30	20	30	30	20
3	30	30	20	30	30	20
4	30	30	20	30	30	20
5	30	30	20	30	30	20

Supplementary Table 2: Dosage dependency experiment. Table rows and columns give treatments, while cell values indicate the number of flies used in that treatment.

Bd Concentration	Male Washed	Male Unwashed	Female Washed	Female Unwashed
0 zsps/fly	8	8	8	8
10^3 zsps/fly	8	8	8	8
10^4 zsps/fly	8	8	8	8
10^5 zsps/fly	8	8	8	8
10^6 zsps/fly	8	8	8	8

Supplementary Table 3: Mortality assay experiment. Table rows and columns give treatments, while cell values indicate the number of flies used in that treatment. Repeated values indicate individual fly vials.

	Bd Positive (10^6 zsps/fly)	**Bd Supernatant**	**Bd Negative (water)**
Male	5 5 5 5	5 5 5 5	5 5 5 5
Female	5 5 5 5	5 5 5 5	5 5 5 5

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