AN ABSTRACT OF THE DISSERTATION OF

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Species declines and extinctions have been recorded across taxa as evidence of an ongoing global biodiversity crisis. Amphibians are at the forefront of these declines with nearly one third of amphibian species estimated to be at risk of extinction. While many factors contribute to population declines and extinctions, the role of disease is of particular concern to amphibians which are hosts to multiple globally distributed pathogens such as the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), the dsDNA virus, ranavirus and trematode parasites, all of which can affect host populations. Amphibian disease-mediated population declines and extinctions have drawn special attention to the relationships between disease and diversity in general, as an understanding of these relationships is crucial for conservation and management efforts.

Host species vary in their competence (effectiveness of transmission) to specific pathogens. As host diversity increases, so does the likelihood of encountering an especially competent or incompetent host, which can alter disease dynamics in the greater community. Prior examinations of amphibian host diversity suggest that increased host diversity reduces disease risks of Bd and trematodes in some systems but the generality of these effect is still

debated. Far less is known about the role of host diversity and ranavirus dynamics. Changes in pathogen diversity also alter disease dynamics, as pathogens interact via their effects on a host and its immune system. In amphibian systems, co-infection often increases disease burdens. although this is not always the case.

Here, using a series of experiments, I examined the interrelationships among diversity and disease in amphibians, with a focus on the Western toad (*Anaxyrus boreas*) as host. Western toads have experienced range reductions and population declines at high altitude sites in the Oregon Cascade Range.

In chapter two I paired laboratory and outdoor mesocosm experiments to examine the relationships of host diversity with ranavirus. In the laboratory experiment I found that after ranavirus exposure, only the Pacific chorus frog (*Pseudacris regilla*) experienced increased mortality when exposed to ranavirus. In the mesocosm experiment, like the laboratory study, the addition of ranavirus exposed conspecifics led to reduced host survival. However, in the presence of *P. regilla* amphibian assemblages experienced nearly complete mortality of all species, including *A. boreas* and the Cascades frog (*Rana cascadae*).

In chapter three I examined the effects of host diversity on Bd disease dynamics. Previous laboratory studies in my system suggested a protective effect of diversity on Bd disease risk. In an outdoor mesocosm experiment I found that communities of only *A. boreas* experienced reduced survival after addition of Bd exposed conspecifics. However when all three host species were present, survival was not different from controls, providing evidence that the reduction of Bd disease risk associated with increased host diversity is robust to changes in scale and ecosystem complexity.

In chapter four I experimentally altered both host and pathogen diversity in a factorial laboratory experiment which elicited a range of responses. I found increased variation in growth in the two days following experimental pathogen exposure, with the direction and strength of the effect modulated by host-pathogen combination. In the absence of other host species *A. boreas*, had increased mortality when simultaneously exposed to both Bd and trematodes.

This dissertation provides evidence of the impacts of diversity on amphibian disease outcomes and highlights the role of community composition in wildlife disease dynamics. This work suggests that to fully appreciate the dynamics of wildlife disease we must consider all interacting species in a community. I provide evidence that increased host diversity may reduce Bd disease risks in a community, while those same changes to host diversity in ranavirus exposed community can lead to collapse of amphibian populations. Further I have shown differences between laboratory and field experiments that suggest pairing diversity studies at different scales is important in the study of wildlife disease.

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Effects of Diversity on Emerging Infectious Diseases of Amphibians

by
Paul W. Snyder

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APPROVED:

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Head of the Department of Integrative Biology

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Paul W. Snyder, Author

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CONTRIBUTION OF AUTHORS

Dr. Andrew Blaustein, my graduate advisor, contributed to the experimental design, analysis and editing of this dissertation. Dr. Cherie Briggs (University of California, Santa Barbara), Dr. Jason Hoverman (Purdue University), Dr. Jason Rohr (Notre Dame University), and Dr. Peiter Johnson (University of Colorado, Boulder) contributed to the experimental design and funding of the experiments which follow, and assisted with editing of Chapter 2. Dr. Daniel Preston (University of Wisconsin-Madison) assisted with collection and identification of trematode parasites and was consulted on analyses. Dr. Carmen Harjoe (Oregon State University) assisted in collection of amphibian eggs and animal husbandry, as well as assisting in mesocosm design. Dr. Chloe Ramsay (Notre Dame University) assisted with experimentation and animal collections as well as leading the laboratory examination in Chapter 2.

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CHAPTER 1 – INTRODUCTION

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Introduction

Accelerated losses in global biodiversity have been recorded across taxa exemplifying a sixth mass extinction event (Wake and Vredenburg 2008, Ceballos and Ehrlich 2009, Ceballos et al. 2010, 2015, 2017, 2020, Barnosky et al. 2011, Pievani 2014) with more than 16,000 species threatened with extinction (IUCN 2020). The IUCN reports that in the past 500 years, human activity accounted for hundreds of species extinctions. Population declines and extinctions have been noted across taxa, with precipitous declines in, amphibians (Blaustein et al. 1994, Fisher and Shaffer 1996, Alford and Richards 1999, Davidson, C 2004, Stuart et al. 2004, Fisher and Garner 2020) birds (Sodhi et al. 2004, Monroe et al. 2019, Rosenberg et al. 2019), corals (Hughes et al. 2017, Sheppard et al. 2020, Pisapia et al. 2020), insects (Didham et al. 2020, Cardoso et al. 2020), large mammals (Cardillo 2005, Spooner et al. 2018, Rosenberg et al. 2019) and reptiles (Gibbons et al. 2000, Alroy 2015) at the global scale. Wildlife population declines and extinctions are, by some estimates, more than 1,000 times the pre-human background extinction rate (Ceballos et al. 2010). Habitat loss and degradation is the most dominant threat to biodiversity, affecting more than 80% of threatened birds, mammals and plants (Singh 2002, Marques et al. 2019, IUCN 2020). Other major anthropogenic threats to global biodiversity include, over-exploitation via hunting, fishing, and other extractions (Lafferty 2004, Allendorf and Hard 2009, Chiyo et al. 2015), invasive and introduced species (Vitousek et al. 1997, Torchin et al. 2003, Schlaepfer et al. 2005, Miaud et al. 2016, Young et al. 2017, Lundgren et al. 2018, Blaustein et al. 2020), pollution, (Lloyd 1992, Arkoosh et al. 1998, Stauffer 2013, Mueller and Schupp 2020) and atmospheric and global climate change (Bancroft et al. 2007, Lawler et al. 2009, Rohr and Raffel 2010, Cook et al. 2013, Oreskes 2018, Spooner et al. 2018). Disease also threatens global biodiversity (Daszak 2000, De Castro and Bolker 2005, Smith et al. 2006, Vredenburg et al. 2010, Hoyt et al. 2016).

As part of the Biodiversity Crisis, amphibian populations are declining worldwide (Blaustein et al. 1994, Fisher and Shaffer 1996, Alford and Richards 1999, Berger et al. 1999, Carey et al. 1999, Kiesecker et al. 2001, Stuart et al. 2004, Vredenburg et al. 2010, Alroy 2015, Grant et al. 2020). Nearly one third of amphibian species are estimated to be at risk of extinction (Stuart et al. 2004). Multiple factors drive these declines and the impact of each factor is varied among species, population, and region (Blaustein et al. 1994b, Alford and Richards 1999, Kiesecker et al. 2001, Blaustein and Kiesecker 2002, Rohr and Raffel 2010, Blaustein et al. 2011, Bradley et al. 2015a, Grant et al. 2020).

Amphibian population declines are especially influenced by disease, caused by a number of different types of pathogens (Daszak et al. 1999, 2003, Chinchar 2002, Robert 2010, Vredenburg et al. 2010, Blaustein et al. 2012, Cohen et al. 2019), including the globally distributed fungal pathogens *Batrachochytrium dendrobatidis* (Bd) (Berger et al. 1998, 1999, Fisher et al. 2009, Rödder et al. 2009, Olson et al. 2013, Scheele et al. 2019, Lambert et al. 2020, Fisher and Garner 2020) and *Batrachochytrium salamandrivorans* (Bsal) (Grant et al. 2016, Stegen et al. 2017, Yap et al. 2017, Health et al. 2018) the globally distributed ranavirus (Chinchar 2002, Greer et al. 2005, Robert 2010, Price et al. 2014) and numerous macro-parasites including trematodes that have differing effects on anatomy and physiology of their amphibian hosts (Johnson et al. 2002, Blaustein and Johnson 2003a, 2003b, Johnson and Sutherland 2003, Johnson et al. 2004, Keeler and Huffman 2009, Rohr, J.R. et al. 2009), shed from infected snails (Sorensen and Minchella 2001, Poulin and Cribb 2002). Some of these trematode parasites such as *Echinostoma spp*. and those in the genus *Ribeiroia* can cause deformities, mortality and may facilitate population level effects (Fried et al. 1997, Blaustein and Johnson 2003a, Rohr, J.R. et al. 2009)

Bd is an aquatic, globally distributed (Fisher et al. 2009, Rödder et al. 2009, Olson et al. 2013), generalist fungal pathogen, that causes the disease, chytridiomycosis, which infects over 40% of amphibian species (Olson et al. 2013). Bd has a complex lifecycle in which the infective stage, motile zoospores, move-toward and encyst-in keratinized tissues of a host (Marantelli et al. 2004, Berger et al. 2005a). The encysted zoospore grows into a sporangium on the skin of the host, forming zoospores (Longcore et al. 1999). Once mature, the sporangia release the motile zoospores into the water where they move toward keratinized host tissue via chemotaxis, to repeat the cycle (Berger et al. 2005a). Bd causes the disease chytridiomycosis, which can result in lethargy, cutaneous erythema, skin sloughing and loss of righting reflex (Longcore et al. 1999, Carey et al. 2006, Fisher et al. 2009, Gahl et al. 2012). Bd can disrupt cutaneous functioning, inhibiting electrolyte transport and deteriorates the electrical functions of the heart, ending in cardiac arrest (Voyles et al. 2009). Bd also has a number of paths to subsistence in the environment such as in the water (Chestnut 2014), soil (Johnson and Speare 2005), and in non-amphibian reservoir hosts such as the crayfish (McMahon et al. 2013, Brannelly et al. 2015, Oficialdegui et al. 2019), and contributes to amphibian population declines and extinctions (Berger et al. 1998, 1999, Skerratt et al. 2007, Rödder et al. 2009, Voyles et al. 2009, Scheele et al. 2019, Fisher and Garner 2020). Numerous experimental studies of Bd have revealed extreme variance in its effects on hosts (Stockwell et al. 2010, Bancroft et al. 2011, Gahl et al. 2012, Gervasi et al. 2013a, Bradley et al. 2015a, Blaustein et al. 2018) Hosts also vary in their susceptibility to Bd throughout their lives, with larvae generally less susceptible to chytridiomycosis than post metamorphic amphibians (Blaustein et al. 2005, Garcia, T. S. et al. 2006, Smith et al. 2007, Han et al. 2008, Gervasi et al. 2014, Langhammer et al. 2014, Bradley et al. 2019b), perhaps because the pathogen only encysts on the keratinized mouthparts of larvae as

opposed to the keratinized skin found in adults (Berger et al. 2005a). However, toxins emitted by Bd can cause mortality in larvae of some species (Blaustein et al. 2005, McMahon et al. 2013). Hosts susceptibility continues to change with the age of the host animal (Carey et al. 1999, Rachowicz and Vredenburg 2004, Haislip et al. 2011, Bradley et al. 2019b). Infection outcomes from Bd are influenced by temperature (Piotrowski et al. 2004, Andre et al. 2008, Hamilton et al. 2012, Bradley et al. 2019a), as the pathogen seems to require a relatively narrow thermal optimum, although there is evidence that changes in temperature may be a stronger predictor of infection outcomes than absolute temperature (Rohr and Raffel 2010, Hamilton et al. 2012, Bradley et al. 2019a). Additional factors such as the identity of the Bd strain (Berger et al. 2005b, Retallick and Miera 2007, Dang et al. 2017), host species diversity (Searle et al. 2011, Becker et al. 2014), community composition (Han et al. 2015), host population (Bradley et al. 2015b) and season (Berger et al. 2004, Andre et al. 2008) impact disease outcomes and transmission. A second emerging, pathogenic chytrid species, *Batrachochytrium salamandrivorans* was identified in salamanders causing similar pathology to Bd (Martel et al. 2013, Grant et al. 2016, Yap et al. 2017).

Ranavirus is an emerging infectious aquatic pathogen of amphibians (Duffus et al. 2015). Ranaviruses are dsDNA viruses in the family Iridioviridae which are capable of infecting fishes, reptiles and amphibians (Chinchar 2002, Chinchar and Waltzek 2014, Brenes et al. 2014, Price et al. 2017). Amphibians can become infected by ranavirus via multiple routes, as the virus is able to invade many cell types (Chinchar 2002, Brunner et al. 2017). Infection can occur from direct contact with an infected individual, exposure to ranavirus contaminated water or via ingestion (Brunner et al. 2007, Brenes et al. 2014). Ranaviruses cause systemic infections which include hemorrhagic lesions of internal organs, skin sloughing and ulceration, although clinical signs are

not always present prior to death (Chinchar 2002).The virus can contribute to amphibian population declines and extinctions (Chinchar 2002, Robert 2010, Price et al. 2014, Earl et al. 2016), and like Bd, ranaviruses have complex disease dynamics. Ranavirus disease dynamics are influenced by temperature (Santos Rojas et al. 2005, Brand et al. 2016), exposure dose (Brunner et al. 2005, Forzán et al. 2015), pathogen strain (Schock et al. 2009) and host identity (Hoverman et al. 2010).

Trematode flatworms may also contribute to amphibian population losses because they can induce anatomical changes that may affect host survival (Johnson 1999, Stopper et al. 2002, Blaustein and Johnson 2003a, 2003b, Rohr, J.R. et al. 2009, Szuroczki and Richardson 2009, Buller 2012). Trematode species of the family *Echinostomatidae* and genus *Ribeiroia* are thought to be both the most prevalent and most able to have population scale impacts on amphibian hosts (Johnson et al. 2004, Rohr, J.R. et al. 2009, Szuroczki and Richardson 2009). Trematode parasites have a complex lifecycle which begins as an egg shed into a body of water via the feces of the definitive host. This egg develops into a freely swimming miracidia and then infects a host snail which serves as first intermediate host. Trematodes reproduce inside the snail, which eventually begins to shed motile cercariae, a free-swimming infective stage of the trematode, that attach to and encyst in a second intermediate host, often amphibians. The encysted cercariae sheds its tail becoming a metacercaria and completes its lifecycle when the definitive host ingests the metacercarial cyst inside an intermediate host (Ginetsinskaya 1988, Sorensen and Minchella 2001, Poulin and Cribb 2002, Galaktionov and Dobrovolskij 2003, Johnson et al. 2004, CDC 2019). Trematode infections may alter host behavior (Daly and Johnson 2011, Szuroczki and Richardson 2012, Koprivnikar et al. 2014, Reynolds and Reynolds 2017), cause limb deformities (Johnson 1999, Johnson et al. 2001a, 2002, Stopper et al. 2002,

Blaustein and Johnson 2003a, 2003b, Schotthoefer et al. 2003b), reduce growth (Schotthoefer et al. 2003a, Keeler and Huffman 2009, Szuroczki and Richardson 2009) and cause mortality in juveniles (Fried et al. 1997, Schotthoefer et al. 2003a, Holland et al. 2007). Trematode species of the family *Echinostomatidae* and genus *Ribeiroia* have different methods of infection which correspond to their increased costs of infection. *Ribeiroia* encyst around the base of the limbs and tail, causing limb malformations in developing animals (Johnson 1999, Johnson et al. 2001a, Kiesecker 2002, Stopper et al. 2002, Blaustein and Johnson 2003a, 2003b, Schotthoefer et al. 2003b, Johnson and Sutherland 2003) and causing mortality in animals which had not yet developed limb buds (Schotthoefer et al. 2003b). *Echinostoma spp.* enter the animal via the cloaca and encyst in the kidneys, reducing growth rates and causing mortality in juveniles (Fried et al. 1997, Schotthoefer et al. 2003a, Holland et al. 2007, Keeler and Huffman 2009, Szuroczki and Richardson 2009). More generally, amphibian immune systems respond differently to macro and micro parasites (Rollins-Smith and Woodhams 2012) which may allow trematode parasites to interact with other pathogens via the hosts immune system (Wuerthner et al. 2017, Koprivnikar et al. 2019).

Diversity and disease:

As global biodiversity continues to decrease, the impact of diversity on disease dynamics has become the focus of recent examinations. It has been suggested that there are predictable patterns dictating the relationship between disease and host diversity (Ostfeld and Keesing 2000). The dilution effect is the expectation that in wildlife systems with a vector borne generalist pathogen and multiple potential host species, disease risk should decrease as the number of potential hosts increase, assuming other factors such as population density remain constant. The mechanisms of dilution can be distilled as: Host species are differentially

competent (effective at transmission of pathogen) to generalist pathogens. As host species richness increases some host species will be less competent and will be poor transmitters for their pathogen, potentially acting as a dead-end host (Begon 2008, Miller and Huppert 2013). Provided all other factors stay the same, changes in transmission from the addition of less competent host species lead to an overall reduction of disease prevalence. This dilution effect is context dependent and is not the only possible interaction between disease and diversity, as no effect, or the opposite effect - the amplification of disease risk with increasing potential host species – is also probable in some systems (Ostfeld and Keesing 2000, 2013, LoGiudice et al. 2003, Keesing et al. 2006, 2006, Randolph and Dobson 2012, Lafferty and Wood 2013, Ostfeld 2013, Randolph S. 2013, Wood and Lafferty 2013, Miller and Huppert 2013, Dinoverm 2013, Huang et al. 2016, Faust et al. 2017, Halsey 2019). The dilution and amplification effects provide insight into complex interactions between a generalist pathogen and its host species. It has become apparent that how a system responds to changes in host diversity is complex and dependent on multiple factors in addition to host competence, such as temperature (Liu et al. 2016), vector host preference (Miller and Huppert 2013), host density (Mihaljevic et al. 2014), community composition (LoGiudice et al. 2003), and scale (Magnusson et al. 2020).

In addition to changing host diversity, pathogen diversity also influences disease dynamics via coinfection (Cattadori et al. 2007, Johnson and Hoverman 2012, Stutz et al. 2018). Hosts can be infected with more than one pathogen species (Sousa 1992, 1993, Levin and Fish 2000, Cattadori et al. 2007, Romansic et al. 2011, Johnson and Hoverman 2012, Kik et al. 2012, Viney and Graham 2013, Ezenwa 2016, Warne et al. 2016, Wuerthner et al. 2017), or more than one strain of a pathogen species (Sharp et al. 1997, Little et al. 1998, Mihaljevic et al. 2018, Lötters et al. 2018, McDonald et al. 2020). Coinfected animals often have disease outcomes

which are different from singly infected animals and coinfections are common in the wild (Ezenwa 2016, Hoarau et al. 2020) and are found in amphibian systems (Romansic et al. 2011, Hoverman et al. 2012, Kik et al. 2012, Warne et al. 2016, Lötters et al. 2018).

Research efforts have increased recently alongside a growing appreciation for the role of coinfection in disease dynamics especially in free-ranging wildlife (Cattadori et al. 2007, Ezenwa, V.O. et al. 2019) and in laboratory studies (Murphy et al. 2011, Wuerthner et al. 2017). Increased pathogen diversity can alter disease dynamics in various ways, including reducing host survival (Johnson and Hoverman 2012, McDonald et al. 2020) and reducing host mortality (Jolles et al. 2008, Wuerthner et al. 2017).

In response to the complexity that considerations of diversity bring to disease dynamics disease ecologists are beginning to synthesize the complex effects of both host and parasite diversity on disease dynamics. Relationships between diversity and disease have been determined to be non-linear and dependent on density and scale (Halliday and Rohr 2019, Rohr et al. 2020). Recently, Halliday's (et al. 2020) metanalysis suggests biodiversity loss may produce the dilution effect. They found the dilution effect to be detectable when changes in biodiversity were disturbance driven but less so in natural biodiversity gradients. Yet, while the literature on disease diversity relationships has grown it is difficult to draw generalized conclusions, renewing calls for standardizations of methods and definitions (Rohr et al. 2020, Teitelbaum et al. 2020, Stewart Merrill and Johnson 2020).

In response to the role of disease in amphibian population declines, amphibian systems have become a major focus for study of diversity and disease. Research into disease-diversity relationships in amphibian systems revealed consistent patterns, with trematode and Bd disease risk diluted by increased host richness (Searle et al. 2011, Johnson et al. 2013, Han et al. 2015,

Civitello et al. 2015). Johnson et al. (2013) proposed a mechanism underlying this dilution effect in amphibians based on extensive surveys of California wetlands: Hosts' naturally aggregated such that competent hosts dominate in low diversity wetlands while the least competent hosts are present only in more diverse wetlands.

While many factors including host identity and community composition can alter the disease/host-diversity relationship, in amphibian systems of the California wetlands species assembled non-randomly such that low diversity wetlands are dominated by competent host species and as diversity increases, and low-competency hosts tend to dominate. This naturally occurring non-random aggregation caused a strong dilution effect with trematode transmission in diverse wetlands reduced over 75% (Johnson et al. 2013). While this pattern has been demonstrated for trematodes, host species are not equally susceptible to all potential pathogens or pathogen strains (Retallick and Miera 2007, Schock et al. 2009, Gervasi et al. 2013b, Dang et al. 2017). Given that a host may be more susceptible to one pathogen than another, a diverse population may dilute disease risk for one pathogen but not for another. Scale (i.e. experimental, local, regional, global) also plays a role in these disease diversity relationships, as what may appear to be a dilution effect at a small scale could vanish at larger scales or vice versa (Cohen et al. 2016). One recent meta-analysis however maintains that the dilution effect holds true regardless of scale (Magnusson et al. 2020).

Here I experimentally examined the disease diversity relationship in sympatric amphibian species of the Oregon cascades mountains and their pathogens with a focus on the Western toad (*Anaxyrus boreas*; previously *Bufo boreas*). *A. boreas* makes an ideal focal species as its populations have declined throughout its range (Muths 2003, Davis and Gregory 2003, Wente et al. 2005, Slough and DeBruyn 2018), it is threatened by disease and by changing climate and

atmospheric conditions (Blaustein et al. 1994, 2018, Kiesecker and Blaustein 1995, Kiesecker et al. 2001, Lawler et al. 2010). In numerous experiments *A. boreas* demonstrated a range of responses to Bd, with low susceptibility in some studies (Carey et al. 2006, Han et al. 2008, Gervasi et al. 2013a) and high susceptibility in others (Garcia, T. S. et al. 2006, Rumschlag et al. 2014), including Blaustein et al. (2005), who demonstrated *A.boreas* mortality in response to the presence of Bd within 48 hours. In addition to mortality, Searle et al. (2014) recorded increased concentration of stress hormones in response of *A. boreas* to Bd infection and in response to both Bd and artificially increased stress hormones, *A. boreas* tadpoles increased in length. Han et al. (2008) recorded increased activity of *A. boreas* when exposed to Bd but animals did not avoid infected conspecifics, nor did they seek out temperatures which may alter disease progression. Little is known about *A. boreas'* relationship to ranavirus, which is a likely cause of some *A. boreas* die-offs (Chinchar and Waltzek 2014). In the only published experimental ranavirus infection of *A. boreas*, the toads were susceptible to ranavirus, experiencing 100% mortality (Earl et al. 2016). *A. boreas* and its trematode parasites also have a long history in the scientific literature with numerous trematode species investigated in western toad hosts, including *Alaria spp*. (Buller 2012), *Echinostoma spp*. which can suppress *A. boreas* stress hormones (Koprivnikar et al. 2019), *Ribeiroia ondantrae,* which causes limb malformations in *A. boreas* (Johnson et al. 2001b) and others (Efford and Tsumura 1969, Ubelaker and Olsen 1972).

In the chapter two I paired laboratory and mesocosm experiments to examine ranavirus disease dynamics. In the laboratory I established individual host responses to ranavirus and in mesocosm experiments I examined the impact of diversity on disease by introducing ranavirus exposed conspecifics to naïve populations of one or three host species in semi-natural outdoor mesocosms. In chapter three I examined the effect of host diversity on Bd disease dynamics in

outdoor mesocosm experiments with populations of one or three hosts. In chapter four I experimentally modified both host and pathogen diversity demonstrating a range of potential effects that changes in diversity can have on disease.

These examinations into the role of diversity in disease dynamics are timely and warranted as global biodiversity is decreasing. Understanding the complexities of disease diversity dynamics will be key to predicting the where, when and how of disease outbreaks, and identifying the relevant factors for future management and conservation efforts. For amphibians, which sit at the forefront of global biodiversity declines, grappling with the complexities of wildlife disease dynamics could be essential for their survival.

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CHAPTER 2 – HOST SPECIES COMPOSITION ALTERS HOST-PATHOGEN DYNAMICS IN A RANAVIRUS-AMPHIBIAN ASSEMBLAGE

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Abstract

Global-scale losses in biodiversity have the potential to alter disease risk through multiple pathways, yet the influence of diversity shifts on host-pathogen dynamics remain controversial. Disease risk from generalist pathogens is especially likely to be affected by changes to host richness. Amphibians represent an ideal system to investigate links among biodiversity and infectious disease because they are experiencing worldwide declines linked to generalist pathogens. To provide predictions for how changes in host species composition affects disease risk, we exposed Pacific chorus frog (*Pseudacris regilla*), Western toad (*Anaxyrus boreas*), and Cascades frog (*Rana cascadae*) tadpoles individually in the laboratory to ranavirus, a generalist pathogen implicated in amphibian mortality events. We then tested the predictions derived from this experiment in outdoor freshwater mesocosms consisting of two host communities, all three amphibian species tested in the laboratory experiment or just *A. boreas*, exposed to ranavirus or sham treated conspecifics. In the laboratory experiment, pathogen exposure reduced the survival of *P. regilla*, while the survival of *A. boreas* and *Rana cascadae* were not affected, suggesting that *P. regilla* might be more susceptible to ranvirus and thus amplify risk from this pathogen to the other species. In support of this hypothesis, greater *A. boreas* mortality occurred in ranavirus-exposed mesocosms containing all three host species than *A. boreas* alone. These results suggest that the susceptibility of Pacific chorus frogs to ranavirus can alter disease dynamics across multiple species, potentially enabling declines in otherwise resistant species.

Introduction

Ongoing losses in biodiversity have led to what is characterized as a sixth major extinction event with the current extinction rate estimated to exceed that of any period across the last 100,000 years (Wilson 1992, Wake and Vredenburg 2008, Ceballos et al. 2015). Loss of biodiversity can lead to changes in community structure, nutrient cycling, and ecosystem production, which can affect the transmission of infectious diseases (Chapin et al. 2000, Butchart et al. 2010, Cardinale et al. 2012, Hooper et al. 2012, Reich et al. 2012). Moreover, changes in diversity may affect the dynamics of disease risk (Ostfeld and Keesing 2000, Johnson et al. 2013, Venesky et al. 2014, Civitello et al. 2015). Disease is associated with many amphibian population declines (Daszak et al. 2003, Muths et al. 2003, Rohr et al. 2008, Blaustein et al. 2012, Grant et al. 2016a, Blaustein et al. 2018). Several globally distributed generalist pathogens have been implicated in large-scale amphibian population die-offs and population declines, including: the chytrid fungi, *Batrachochytrium dendrobatidis* (Bd) (Berger et al. 1998, Voyles et al. 2009, Scheele et al. 2019) and *Batrachochytrium salamandrivorans* (Bsal) (Grant et al. 2016b, Health et al. 2018) and the *iridovirus*, *Ranavirus* (Gray et al. 2009, Price et al. 2014, Duffus et al. 2015).

Examinations of the relationship between species richness and disease risk have highlighted the importance of host competence, the ability of a host to maintain and transmit an infection, in multi-host disease systems (Kilpatrick et al. 2006, Thieltges et al. 2011). Studies examining the relationship between amphibian diversity and disease risk have reported reductions in disease risk with increased host richness (Searle et al. 2011, Venesky et al. 2014, Han et al. 2015). Relationships between amphibian host diversity and disease risk have not been experimentally examined with respect to ranavirus.

Ranaviruses are globally distributed, generalist aquatic viruses, which can infect amphibians, fishes, and reptiles (Gray et al. 2009, Brenes et al. 2014). Ranaviruses can cause hemorrhaging, organ failure, and death in hosts (Gray et al. 2009). Experiments have revealed that ranavirus susceptibility and pathology vary with host species and viral strain, such that mortality in ranavirus-infected species ranges from 0% to 100% (Cullen and Owens 2002, Hoverman et al. 2010, 2011, Earl et al. 2016, Blaustein et al. 2018). It is unknown how individual host competencies in this multi-host-disease system affects transmission and outcomes of infections in amphibians. Thus, we examined the susceptibility of three sympatric amphibian species found in Oregon's Cascades mountains, Western toads (*Anaxyrus boreas*), Pacific chorus frogs (*Pseudacris regilla*), and Cascades frogs (*Rana cascadae*) to ranavirus infection in laboratory experiments where tadpole of each host were exposed to ranavirus individually. This provided hypotheses for how communities of these hosts should affect ranviral disease risk. To test these predictions, we conducted an outdoor freshwater mesocosm experiment that crossed two host communities, all three amphibian species tested in the laboratory experiment or just *Anaxyrus boreas*, with exposure to ranavirus or sham treated conspecifics.

Materials and Methods

Animal collection and husbandry:

Amphibian eggs were collected from naturally occurring breeding sites in Oregon in January 2015. Eggs of *P. regilla* were collected in the Willamette Valley at two sites (44.572°N, -123.300°W and 44.691°N, -123.216°W). Eggs of *R. cascadae* were collected at Parish Lake (44.522°N, -122.031°W) while those of *A. boreas* were collected in the Deschutes National Forest (44.032°N, -121.687°W). We housed eggs in 40 L aquaria filled with dechlorinated water

treated with AquaNova (Kordon LLC, Hayward CA, Item # 31161) and Amquel (Kordon LLC, Hayward CA, Item # 31261). Within two days of hatching, we moved larval amphibians into 40 L aquaria (treated as above) at densities of 1-2 animals per L and fed animals a mixture of rabbit chow, spirulina flakes, and shrimp flakes (3:1:1) *ab libitum* every other day. Complete water changes occurred weekly and temperatures remained between 12°C and 15°C.

Ranavirus culture:

We used a *Ranavirus* isolate obtained from infected Wood frog tadpoles (*R. sylvatica*) collected from Iron River, Michigan. The virus was cultured using a protocol adapted from Hoverman et al. (2010) wherein virus was passaged through fathead minnow cells incubated at 28°C without CO_2 and fed with Eagle's minimum essential medium with Hank's salts (HMEM) and 5% fetal bovine serum. The virus was stored at -80°C until the start of the experiments. The isolate was on the third passage since original isolation.

Laboratory experimental design:

To test susceptibility of host species to ranavirus, we experimentally exposed individual *P. regilla*, *A. boreas*, and *R. cascadae* to virus or a sham control. Experimental units were 1000 mL containers filled with 800 mL of water, with one animal per container. For the virus treatment, animals were exposed to a total of 10^5 plaque forming units (PFU), which equated to a concentration of 10^2 PFU per mL. Previous studies have used concentrations from 10^2 to 10^6 PFU per mL and demonstrated sublethal effects on amphibians (Hoverman et al. 2010, 2011). Control animals received 500ul of HMEM without virus as a sham treatment. Each treatment included 28 animals (n=28) for each of the three species. Forty-eight hours prior to exposure, amphibians between Gosner developmental stages 26-30, determined by hind limb bud

development (Gosner 1960), were moved to individual 1000 mL containers with 800 mL of water and acclimated to 23°C. For the duration of the experiment, water was changed every 4 days. At Gosner stage 42 (determined by the emergence of front limbs), animals were moved to individual plastic containers (33.5cm x 20cm x 9cm) tilted at an approximately 30° angle to create a partially dry environment. These containers were secured with a mesh lid and elastic. Post-metamorphic amphibians were fed 3-5 vitamin and mineral dusted crickets *ad libitum.* Animals were checked twice daily for mortality, upon death, the date was recorded and the animal frozen and stored at -20°C. Animals surviving 3-weeks post-metamorphosis were euthanized and considered to have survived. Animals were euthanized via submersion in a neutrally buffered MS-222 solution and were then frozen and stored at -20°C. Quantitative PCR was used to determine Ranavirus loads, from livers of euthanized animals (Picco et al. 2007)

Mesocosm experimental design:

To test how ranavirus transmission and mortality varied within single- and multiple- host-species assemblages, we used 120L mesocosms at Oregon State University's Lewis-Brown Horticultural Farm (44.548°N, -123.215°W). Mesocosms were lined with sterilized leaf-litter covering the bottom of the tank, filled with well water and inoculated with 1L of water containing zooplankton, phytoplankton and periphyton prepared in artificial ponds, then covered with mesh lids. Mesocosms were left for four weeks to establish algal growth prior to the onset of the experiment. Mesocosms included two levels of host composition: 1-host (*A. boreas* only, 36 tadpoles) or 3-hosts (*P. regilla, R. cascadae* and *A. boreas*, with 12 tadpoles of each species). *A. boreas* was chosen for the 1-host treatments because they are the most abundant and dominant amphibian species in high elevation sites in our Oregon study system. Thus, this reference condition reflects the fact that this species is widespread and would most likely be lost last from

these systems. Our design was substitutive with a total of 36 tadpoles in every treatment. Hosts were exposed to either three ranavirus-exposed animals (ranavirus treatment) or three naïve animals (sham-inoculated control) as shown in Figure 1. Ranavirus exposures occurred in 10L treated water to which either ranavirus or a sham solution was added. Ranavirus, was thawed and added to the 10L aquaria to a concentration of 10^3 PFU/ml. Five ml sterile HMEM was added to control treatments. Animals were moved from the laboratory to the mesocosm location in 2-liter collection jars.

Each of our four treatments was run with four replicates (16 total mesocosms; Fig 1). Thirty-three naïve larvae were added to each mesocosm on day zero; on day one of the experiment, we added an additional three animals to each mesocosm, having been exposed either to ranavirus or the sham inoculum as described above. At the experiment's onset, all animals were larvae; *A. boreas* (Gosner stages 25-28), *P. regilla* (Gosner stages 25-30) and *R. cascadae* (Gosner stages 25-28). Well water was added to mesocosms weekly to offset evaporation. Dead animals were not removed from mesocosms as dead conspecifics may be an important infection source (Hoverman et al. 2010, Miller et al. 2011). Foam floats were placed in mesocosms to allow metamorphosing animals to leave the water. On day 30, we measured survival by catching and counting each animal, briefly housing them in a 10L aquaria of water from the same mesocosm, then returning them immediately to mesocosms. On day 40, we removed and euthanized all metamorphic animals, which in some replicates risked becoming too numerous to be accommodated by our mesocosms. On day 60, we terminated the mesocosm experiment by collecting and euthanizing, as above, all remaining animals including larvae and metamorphs.

Analysis:

To examine survival in the laboratory experiment, we used Kaplan-Meier survival curves to display survival in ranavirus-exposed and control individuals of each species and survival analysis was performed in JMP 14.1 (*JMP* 2019) using the Cox-Mantel test (Mantel and Haenszel 1959, Cox 1972). For *P. regilla*, Log ranavirus load was compared to survival via simple linear regression after removing euthanized animals (Rohr et al. 2010). This analysis was not completed on other hosts as their viral loads were too low for quantification. For each timepoint (30 and 60 days) a two-way ANOVA was used to test for main effects of virus- and richness-treatments and their interaction on the survival of *A. boreas* and the on total mesocosm survival.

Results

In the laboratory experiment, *P. regilla* survival was significantly reduced by ranavirus exposure ($P < 0.001$, Fig 2A), with ranavirus-exposed *P. regilla* surviving a mean of 7.32 days (SE = 0.56), compared with 31.11 days (SE = 1.12) for controls. *A. boreas* and *R. cascadae* survival was not significantly different between ranavirus and control treatments (Fig 2B, Fig 2C). In our tolerance analysis of *P. regilla*, ranavirus load was not correlated with host survival time (Fig 3). *A. boreas* viral loads were too low for quantification and despite exposure, no viral loads were detected in *R. cascadae*.

In the mesocosm experiment, there was a significant interaction between the effect of virus and host richness on survival of *A. boreas* at days 30 (ANOVA $F_{1,12} = 43.89$, $P < 0.0001$) and 60 (ANOVA $F_{1,12} = 30.74$, $P = 0.0001$). Survival was reduced relative to controls in treatments that included both virus exposure and high richness (3-hosts). We observed a similar statistical interaction between virus exposure and host richness when considering total host

survival (i.e., the combined survival of all three hosts) at days 30 (ANOVA $F_{1,12} = 122.18$, $P < 0.0001$) and 60 (ANOVA $F_{1,12} = 32.25$, $P = 0.0001$).

Discussion

In our laboratory experiment, ranavirus-exposed *P. regilla* had significantly reduced survival relative to the control treatment, with most of the mortality occurring in the first 10 days post-exposure (Fig 2A), while *A. boreas* and *R. cascadae* survival did not differ from controls (Fig 2B, 2C). This suggests that, of these three co-occurring species, only *P. regilla* is especially prone to ranaviral-induced mortality. Despite the susceptibility of *P. regilla* to ranavirus, the post-infection survival time appears to be independent of the host's viral load (Fig 3).

In the mesocosm experiment, we found that ranavirus mortality changed as species composition changed with increased mortality in the 3-host species relative to the 1-host species treatment. In the high richness treatments, survival of *A. boreas* and total mesocosm survival followed the same pattern (Fig 4), suggesting similar mortality across species in the 3- and 1-host species treatments despite differences in host susceptibility demonstrated in the laboratory experiment. In contrast, experimental and observational amphibian studies have consistently found that increased host richness dilutes disease risk of amphibian pathogens and parasites (Searle et al. 2011, Venesky et al. 2014, Han et al. 2015, Civitello et al. 2015).

Our mesocosm results may be attributed to differences in host identity among the treatments (susceptibility/competence) and the way in which our experimental communities were structured (Johnson et al. 2013, Rohr et al. 2020). In the presence of the susceptible host *P. regilla* in the mesocosm experiment, ranavirus mortality for all three species was nearly 100%. There is evidence that greater viral exposure than used in our experiments can cause 100%

mortality in *A. boreas* (Earl et al. 2016) and that dose affects ranavirus infection outcomes more generally (Brunner et al. 2005). Given that *A. boreas* survival did not differ significantly from controls in our laboratory experiment, the higher mortality in the mesocosm experiment's 3-host treatment suggests that *A. boreas* were exposed to more viral particles than in the 1-host treatment, potentially generated by the presence of the highly susceptible host *P. regilla*. Here, the addition of a host species that is more susceptible than our focal host, *A. boreas*, likely drove the increased mortality in our high richness treatment. Thus, this is an effect of species composition and we cannot conclude that this is driven by a change in richness independent of the compositional change. Hence, the relationship we found between species richness and ranavirus outcomes might be expected to change had we selected a different single-host species for comparison. If we had assembled regimes with the ranavirus-susceptible *P. regilla* in our 1-host treatments, we likely would have seen a dilution of disease risk when we added *A. boreas* and *R. cascadae*.

In our study system, the host species most likely to dominate a given wetland is based, in part, on elevation. *A. boreas* tends to dominate in the higher elevations sites where we work. These results give us some idea of how a change in host species richness or community composition might alter the course of ranavirus outbreaks in *A. boreas* dominated wetlands. In contrast, at lower elevations, *P. regilla* tends to dominate, and thus future studies should assess whether the likelihood of dilution or amplification changes with altitude.

For species experiencing disease-driven population declines, understanding the dynamics of decreasing biodiversity and disease risk is of paramount interest. The dilution effect hypothesis suggests that biodiversity can reduce disease risk (Ostfeld and Keesing 2000, Johnson et al. 2013, Civitello et al. 2015) and both observational and experimental studies have

demonstrated this relationship in amphibian disease systems (Searle et al. 2011, Venesky et al. 2014, Han et al. 2015). Here we demonstrate the importance of host competence and species composition as key factors in understanding the relationships between diversity and disease. Our experimental results highlight how host identity and changes in community composition could factor into disease-associated population die offs and potential population declines.

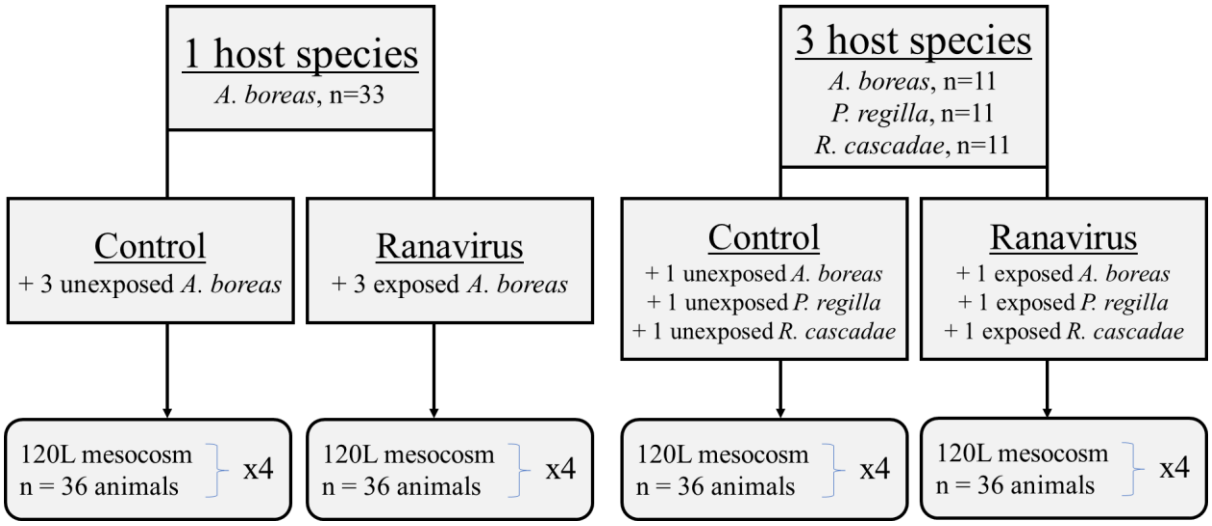
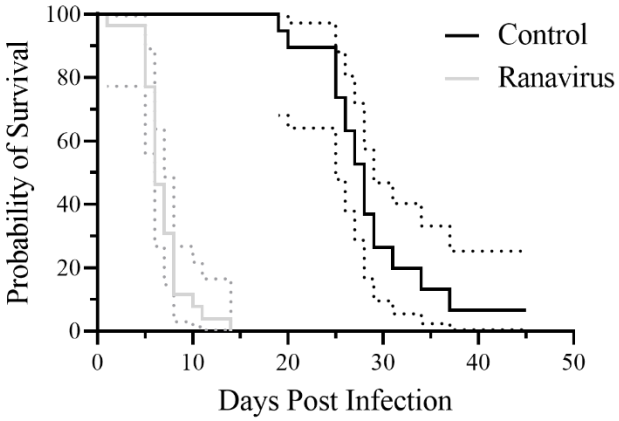
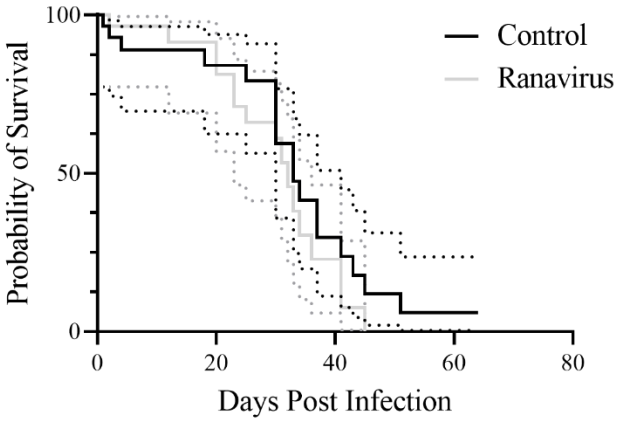


Fig 1. Visual representation of mesocosm experimental design comparing the effects of ranavirus exposure on survival at two levels of host richness.

A - *P. regilla*



B - *A. boreas*



C - *R. cascadae*

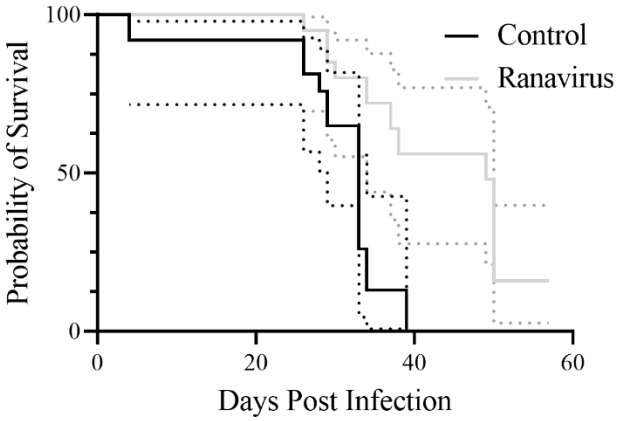


Fig 2. Survival curves for *Pseudacris regilla* (A), *A. boreas* (B), and *R. cascadae* (C), comparing ranavirus exposed (grey) and control (black) individuals. 95% confidence intervals represented by grey and black dotted lines, respectively. Survival was significantly decreased in ranavirus exposed *P. regilla* (A).

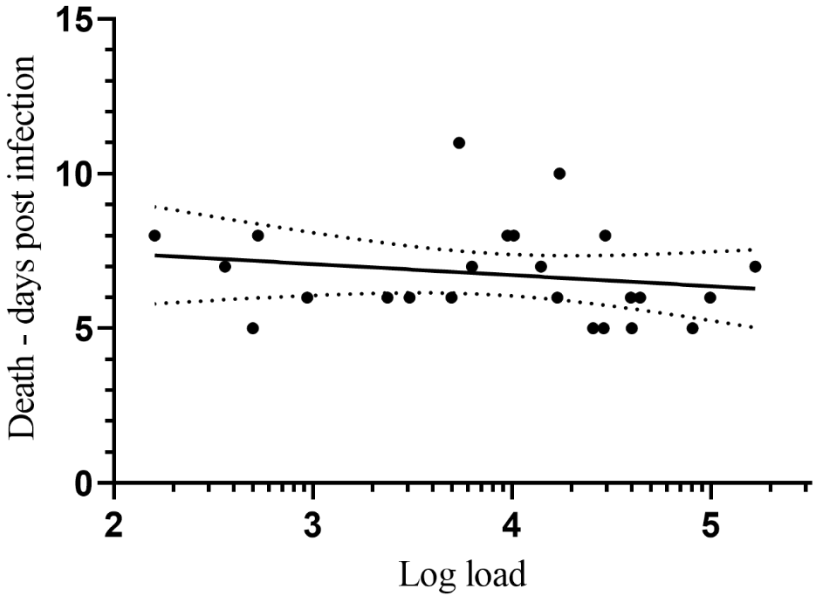


Fig 3. Number of days post ranavirus infection that ranavirus exposed *Pseudacris regilla* died compared to the ranavirus load, in genome equivalents, at death. Euthanized animals are not included. The slope of the trendline is not significantly different from zero.

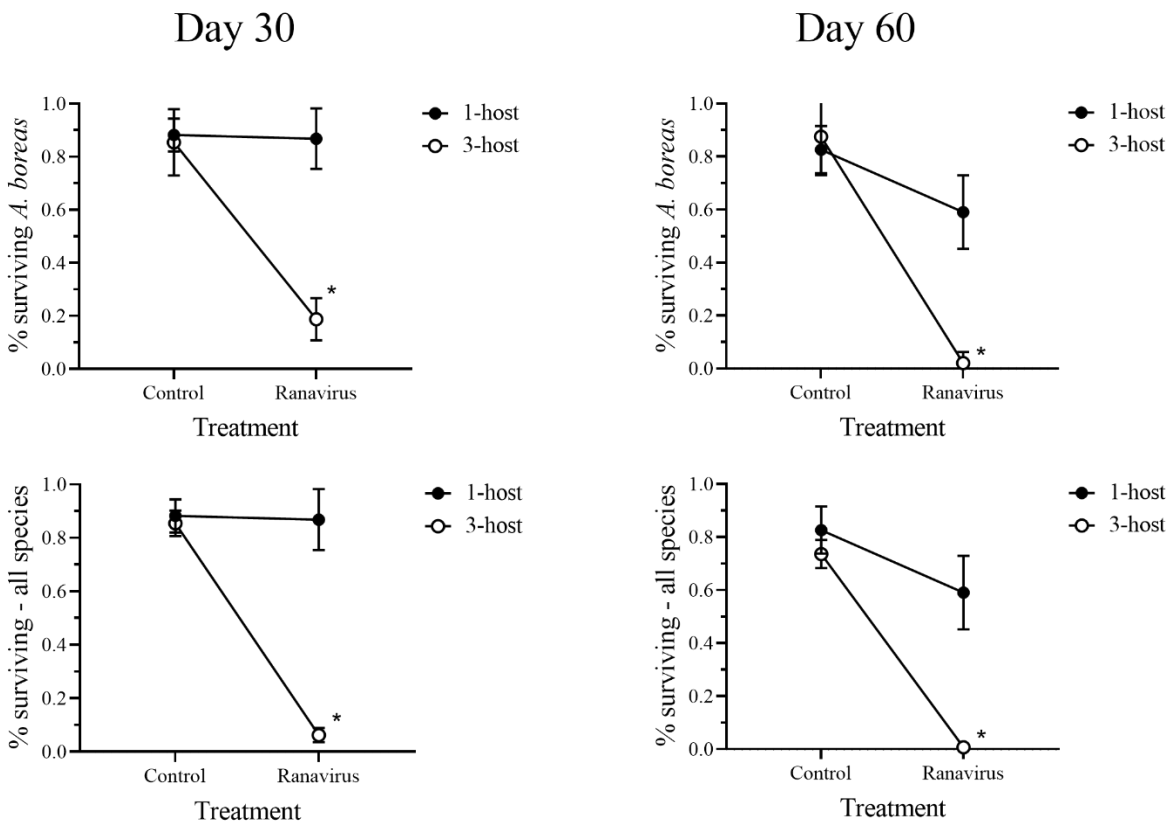


Fig 4. Mean proportion *A. boreas* (top) and total mesocosm survival by treatment at 30 and 60 days. Error bars represent standard deviation. Statistically different means are marked with an asterisk (*).

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CHAPTER 3 – SPECIES COMPOSITION OF AMPHIBIAN ASSEMBLAGES AFFECTS WESTERN TOAD *(ANAXYRUS BOREAS)* SURVIVORSHIP WHEN EXPOSED TO THE FUNGAL PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS*.

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Pieter T. J. Johnson, Daniel L. Preston, Jason R. Rohr and Andrew R. Blaustein

Abstract

Ongoing reductions in biodiversity have highlighted the impact of host species diversity on disease outcomes in systems where an individual pathogen can infect multiple host species in a community. The relationships among host diversity and disease are complex and context dependent. Here we focused on a generalist fungal pathogen of amphibians, *Batrachochytrium dendrobatitis* (Bd) and its relationship to host diversity in amphibian assemblages. Bd is globally distributed and is implicated in worldwide amphibian population declines. Amphibian risk to Bd exposure is influenced by numerous biotic factors including host identity, developmental stage, age, and the number of host species present. Previous laboratory research showed that in the presence of Bd, greater host diversity was associated with increased host survival. We examined this relationship at a larger scale in simulated outdoor ponds in which Bd exposed amphibians were added to a naïve assemblages of potential host amphibians. Using *Anaxyrus boreas* as our focal host species, we found additional evidence to support a reduction of Bd disease risk with increased host diversity.

Introduction

Rapid, global losses in biodiversity suggest that we may be experiencing a worldwide mass extinction event (Wake and Vredenburg 2008, Ceballos et al. 2015). The causes of biodiversity losses are complex and context dependent (Morris and Heidinga 1997, Sax and Gaines 2003, Krauss et al. 2010). Amphibians are at the forefront of this dynamic as their populations are declining worldwide and undergoing both local population declines and species level extinctions exceeding those of birds and mammals (Stuart et al. 2004, Alroy 2015). Like other taxa, amphibians populations are affected by multiple factors including atmospheric and climate change, habitat destruction, over-harvesting, invasive species and infectious disease (Alford and Richards 1999, Wake and Vredenburg 2008, Blaustein et al. 2011, 2018, Buck et al. 2012). Disease plays an especially important role in the declines of amphibian populations (Daszak et al. 1999, Crawford et al. 2010, Blaustein et al. 2018). Globally distributed emerging infectious diseases such as the fungal pathogens *Batrachochytrium dendrobatidis* (Bd), *B. salamandrivorans* and the aquatic Ranavirus have been identified as primary drivers in numerous amphibian population declines (Skerratt et al. 2007, Chinchar et al. 2009, Kik et al. 2011, Olson et al. 2013).

Bd, which causes the disease chytridiomycosis (Daszak et al. 1999, Fisher 2009), has been implicated in numerous amphibian population declines and affects more than 700 species (Berger et al. 1998, Crawford et al. 2010, Lips 2016). Bd grows on keratinized tissues, which include the mouthparts of larval amphibians and the skin of post-metamorphic frogs (Berger et al. 2005, Greenspan et al. 2012). Symptoms of Chytridiomycosis include: lethargy, skin sloughing, loss of righting reflex and eventually cardiac arrest (Voyles, J. et al. 2007, Voyles et al. 2009). Infection outcomes vary based on numerous factors including host identity (Tobler and

Schmidt 2010, Bancroft et al. 2011, Gervasi et al. 2013, 2017, Bradley et al. 2015), pathogen strain (Berger et al. 2005, Retallick and Miera 2007, Schock et al. 2009, Dang et al. 2017) environmental conditions (Berger et al. 2004, Rohr and Raffel 2010, Buck et al. 2011, Hamilton et al. 2012, Urbina and Benavides 2015) host age and developmental stage (Lamirande and Nichols 2002, Hanlon and Parris 2014, Echaubard et al. 2016, Pochini and Hoverman 2017, Garcia et al. 2017, Bradley et al. 2019b) and the species composition of amphibian assemblages (Searle et al. 2011, Johnson et al. 2013, Venesky et al. 2014, Han et al. 2015).

Although numerous studies on the effects of Bd have been conducted, the majority of studies only consider a single host species when investigating the effects of Bd (Blaustein et al. 2018). Single species studies may not fully describe Bd- host dynamics as the composition of amphibian assemblages may influence disease spread and other disease dynamics. For example, recent studies have shown that increased host diversity dilutes disease risk for trematode parasites (Johnson et al. 2013, Wuerthner et al. 2017). Since amphibians generally share their habitat with other amphibians, it is important to understand the dynamics of Bd-host interactions in systems with multiple species.

Here we used a mesocosm experiment to examine how Bd affects disease dynamics in a focal species in assemblages where host species diversity is manipulated. The western toad (Anaxyrus *boreas*) is an ideal focal species as its populations have declined throughout its range (Muths et al. 2003, Davis and Gregory 2003, Wente et al. 2005, Slough and DeBruyn 2018) and it has experimentally demonstrated interesting variation in Bd susceptibility; with high susceptibility in some studies (Blaustein et al. 2005, Garcia, T. S. et al. 2006, Rumschlag et al. 2014) and low susceptibility in others (Carey et al. 2006, Han et al. 2008, Gervasi et al. 2013). Previous research in this system showed that diversity dilutes disease risk of Bd (Searle et al.

2011, Venesky et al. 2014, Han et al. 2015) Therefore, we expected toads in assemblages with multiple hosts exposed to Bd would have greater survival than those in assemblages of *A. boreas* alone. Unlike previous studies that were conducted in the laboratory, our study incorporated semi-natural mesocosms in the field where amphibians were subjected to natural abiotic parameters and different biotic aspects that will be outlined below. Also separating our work from previous efforts is exposure route, previous examinations exposed the entire environment to the pathogen. Here we aim to mimic the natural arrival of the pathogen by adding infected conspecifics to a naïve population. Although comparing results between studies using different experimental protocols is often difficult to interpret (Blaustein et al. 2018), such comparisons often shed light on the generality of results obtained from different studies.

Materials and Methods

Animal collection and husbandry:

Amphibian eggs were collected from naturally occurring breeding sites in Oregon in January 2015. Eggs of the Pacific treefrog (*Psuedacris regilla*) were collected in the Willamette Valley at two sites (44.572°N, -123.300°W and 44.691°N, -123.216°W). We collected Cascades frog (*Rana cascadae*) eggs at Parish Lake (44.522°N, -122.031°W) while eggs of Western toads (*A. boreas*) were collected in the Deschutes National Forest (44.032°N, -121.687°W). We housed amphibian eggs in 40 L aquaria filled with dechlorinated water treated with AquaNova (Kordon LLC, Hayward CA, Item # 31161) and Amquel (Kordon LLC, Hayward CA, Item # 31261). Within two days of hatching, we moved larval amphibians into 40 L aquaria (treated as above) at densities of 1-2 animals per L and fed animals a mixture of rabbit chow, spirulina

flakes, and shrimp flakes (3:1:1) *ab libitum* every other day. Water changes occurred weekly and temperatures was maintained between 12°C and 15°C.

Batrachochytrium dendrobatidis:

Bd was cultured from the JEL-646 isolate (isolated form P. regilla in Point Reyes, California; 38.178°N, -122.991°W), obtained from Joyce E. Longcore, University of Maine. Isolates were grown first in a 1% tryptone broth until growth was visible. One mL of the Bd-tryptone broth was plated onto 100mm culture dishes containing 1% tryptone agar 1 to 2 weeks prior to experimentation. For experimentation, Bd zoospores were washed off agar plates with a rubber spatula and 10mL of water, were pooled and quantified via hemocytometer at 400X. Zoospore solution was added to 10L aquaria for a final concentration of 10^3 zoospores/mL. In the sham treatment uncultured agar plates were washed with 10ml water and the solution was added to a 10L aquaria. Animals were exposed to either Bd or sham by being moved into the 10L aquaria with either Bd or sham treatment for 30 hours. After exposure, animals were moved in 2L collection jars to the mesocosm site and added to communities of naive animals by treatment.

Experimental Design:

To test how exposure to Bd-infected conspecifics altered outcomes within single- and multiple- host-species assemblages, we used 120L mesocosms at the Oregon State University Lewis-Brown Horticultural Farm (44.548°N, -123.215°W). Mesocosms were lined with autoclave-sterilized leaf-litter to cover the mesocosm floor, filled with well water and inoculated with 1L of water containing zooplankton, phytoplankton and periphyton from outdoor artificial ponds and then covered with secured mesh lids. Prior to experimentation, mesocosms were allowed four weeks to establish algal growth. Mesocosms included two levels of host richness: 1-host (*A. boreas*) or 3-hosts (*P. regilla, R. cascadae and A. boreas*). Naïve hosts were exposed

to either three Bd-exposed conspecifics (Bd treatment) or three naïve conspecifics (control) (Figure 1). Bd exposures occurred over 30 hours in 10L treated water to which either Bd or a sham solution was added.

Each of four treatments was run in four replicates (16 total mesocosms; Figure 1). Thirty-three unexposed larvae were added to each mesocosm on day zero and on day one three additional animals (sham or ranavirus exposed) were added by treatment. At the experiment's onset, all animals were larvae at Gosner (1960) developmental stages 25-28); *A. boreas* (Gosner stages 25-28), *P. regilla* (Gosner stages 25-30) and *R. cascadae* (Gosner stages 25-28). Water levels were checked and maintained weekly to offset evaporation. Bd grows on, and can be isolated and cultured from dead amphibian skin suggesting a potential source of infection (Garner et al. 2005, Walker et al. 2007), therefore dead animals remained in our experiment as a potential source of infection. Floats made of foam and mesh-wire were placed in mesocosms to allow metamorphosing animals to leave the water. On day 30, we quantified survival by species, catching and counting each animal. On day 40, we began removing and euthanizing metamorphic animals, which in some replicates risked becoming too numerous to be accommodated by the floating platforms in our mesocosm design. On day 60, we terminated the mesocosm experiment by collecting, swabbing and euthanizing all remaining animals. Animals were swabbed based on developmental stage; larvae were swabbed orally as Bd encycsts on their keritanized mouthparts. The ventral surface of the legs of metamorphs were swabbed. Animals between these two stages were swabbed both orally and on the ventral surface of their legs. Swabs were labelled and stored at -20°C until they were shipped on ice to Cheryl Briggs at University of California, Santa Barbara for Bd quantification.

Analysis:

To examine *A. boreas* survival in response to Bd- and diversity-treatments we used a two-way ANOVA to test for main and interaction effects at 30 and 60 days. Total mesocosm survival was also examined via two-way ANOVA testing for main effects of Bd- and diversity-treatments and their interactions.

Bd load was analyzed via qPCR at the University of California, Santa Barbara, using established methods (Boyle et al. 2004).

Results

A. boreas survival at 30 days was not altered significantly by treatment. At 60 days there was a significant interaction between the effect of Bd and host diversity on *A. boreas* survival (ANOVA $F_{1,12} = 29.93$, $P = 0.0003$). There were no differences by day 30 in total mesocosm survival, but there was a significant interaction effect on survival at 60 days (ANOVA $F_{1,12} = 5.20$, $P = 0.0417$). Whether considering *A. boreas* survival alone or total mesocosm survival, survival was reduced in 1-host Bd treatments but not in 3-host Bd treatments (Figure 2).

None of the surviving animals swabbed for the presence of Bd zoospores tested positive.

Discussion

In our mesocosm experiment adding Bd-exposed conspecifics to naive populations of one- or three-host species had no effect on survival at 30 days but by 60 days survival was reduced in the one-host Bd treatment compared with controls. Survival was not significantly impacted in the three-host Bd treatment. These results suggest a protective effect from increased host diversity and suggest that *A.boreas* benefitted from the effects of increased host diversity.

Two months after the addition of Bd-exposed conspecifics, survival in *A. boreas* only assemblages dropped to 42%. This suggests *A. boreas* is susceptible to Bd infection and that

impacts on host survival in assemblages may vary with time in different systems. In our study, the delayed effect of Bd exposure on *A. boreas* host survival was outside the timeframe of most experimental examinations of *A. boreas* and Bd, which often use an experimental timeline of 15 to 30 days (Blaustein et al. 2005, Garcia, T. S. et al. 2006, Han et al. 2008, Gervasi et al. 2013, Searle et al. 2014). In our experiment, community exposure to Bd came from exposed conspecifics, while previous examinations have utilized direct infections. This difference in exposure method prevents us from comparing exposure dose directly but it is possible that exposures from infected conspecifics are of a lower dose than those used in direct infections (10^3 to 10^7 zoospores).

Our study supports other research showing that disease risk to Bd decreases with increasing host diversity (Searle et al. 2011, Han et al. 2015). Previous studies with the same species were conducted in the laboratory (Searle et al. 2011, Han et al. 2015) while our study was conducted in semi-natural mesocosms subjected to natural abiotic and biotic parameters. Additionally, the density of animals used and the scale of the laboratory studies differed greatly from our mesocosms experiment. Our study used indirect transmission of Bd whereas the previous laboratory studies added Bd to the system directly (Searle et al. 2011; Han et al. 2015). Given these key methodological differences in other studies of the same system - including differences in venue and both abiotic and biotic variables - taken together the overall conclusion that increased species diversity reduces disease susceptibility is even more robust in this system.

None of the animals in our experiment tested positive for the presence of Bd. There are several possible reasons for this result. Foremost, as dead animals were left to decompose in mesocosms as a potential pathogen source, as such only surviving animals were tested, selecting against the testing of animals with the highest loads. It is also possible that the surviving animals

tested were either never infected or cleared their infection prior to the end of the 60 day experiment. Water temperatures in this experiment ranged from 18°C to 26°C, as temperature is one of the most important abiotic factors identified for Bd, it is also possible that the warmer temperatures slowed Bd growth or facilitated the clearing of infections (McMahon et al. 2014, Bradley et al. 2019a). Nevertheless, animals exposed in one-host treatments showed reduced survival compared with controls. Host composition can significantly alter disease outcomes, as demonstrated here and should be a consideration in experimental examinations of generalist pathogens.

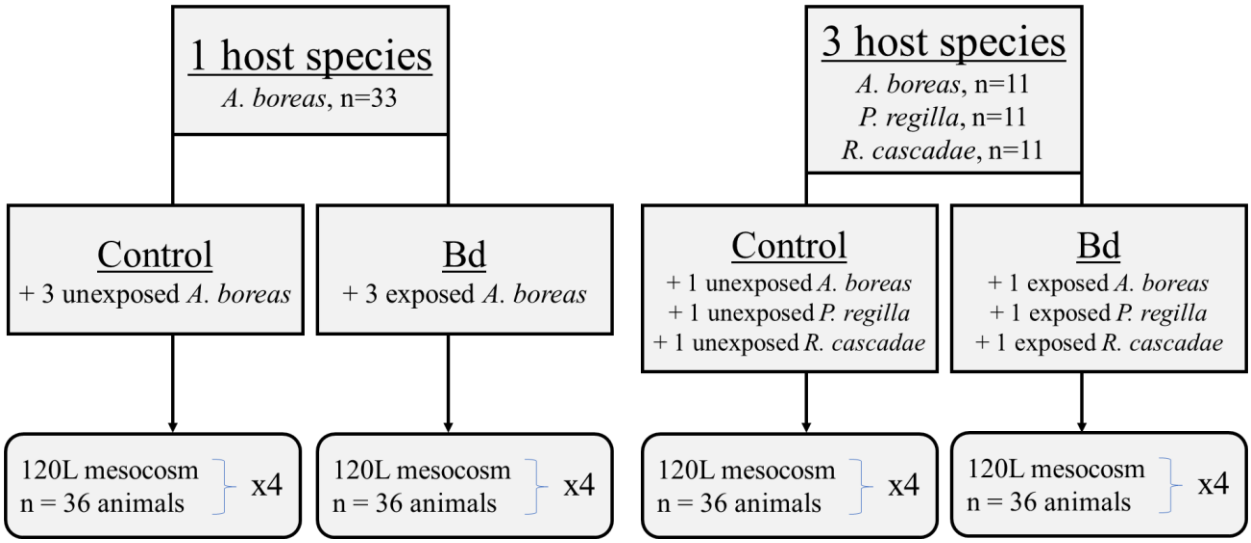

Fig 1. Mesocosm experimental design comparing the effects of *Batrachochytrium dendrobatidis* exposure on survival at two levels of host diversity.

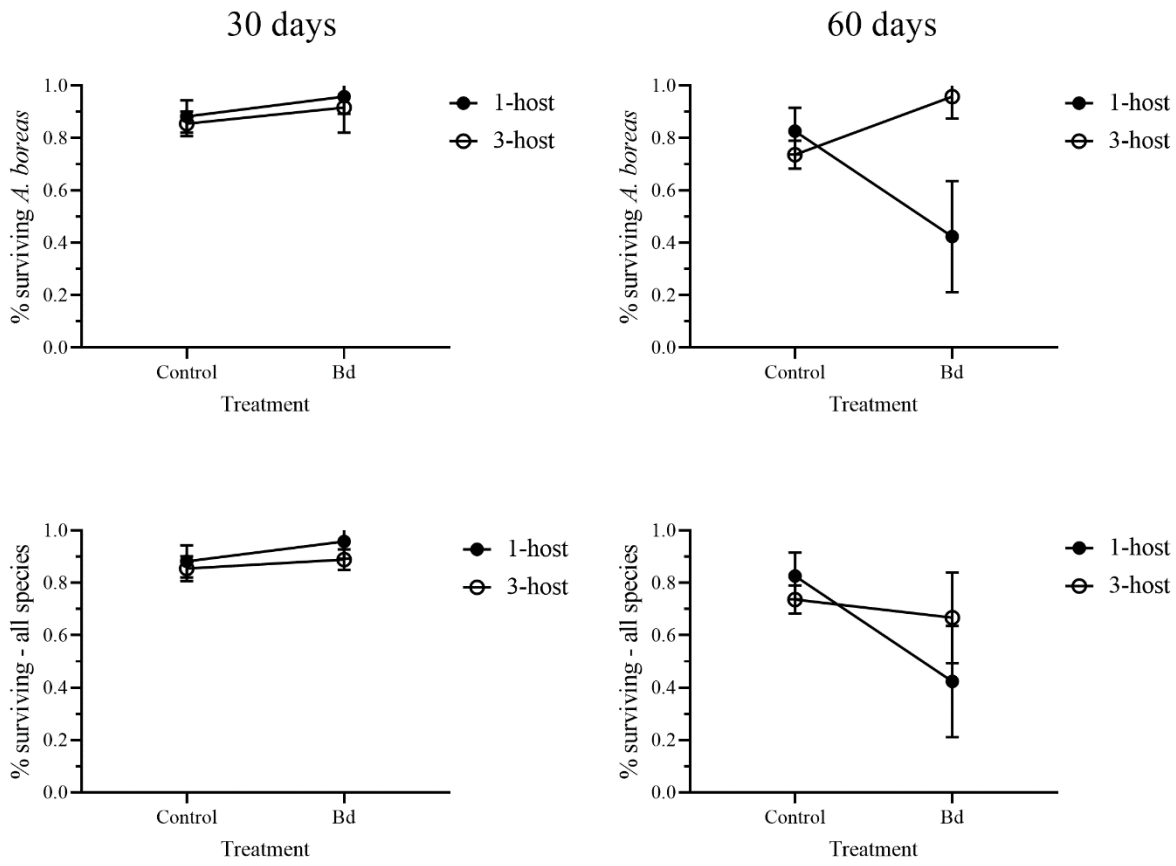

Fig 2. Mean survival of *A. boreas* (top) and all species combined (bottom) by treatment at 30 and 60 days.

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CHAPTER 4 - HOST-PATHOGEN IDENTITY AND DIVERSITY EFFECTS ON AMPHIBIAN HOSTS

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Abstract

Numerous factors influence wildlife disease dynamics including environmental conditions, community structure and the life histories of interacting hosts and pathogens. Global biodiversity decline continues to reveal important relationships between species diversity and disease dynamics. For amphibians, a group currently experiencing disease mediated global population declines, this relationship is vital for conservation efforts. Here we experimentally examined disease dynamics in a system with three amphibian host species and three generalist pathogens, a fungus (*Batrachochytrium dendrobatidis*), ranavirus and trematode parasites. We exposed naïve populations of one or more host species to combinations of infected/uninfected conspecifics or trematode cercariae. Our focal host species the Western toad (*Anaxyrus boreas*) had increased mortality when exposed to both *Batrachochytrium* and trematodes over fourteen days. *A. boreas* also demonstrated variations in growth in the first two days of the experiment with the direction and magnitude of changes determined by treatment combination. Our results highlight the complexity of wildlife disease dynamics and reinforce the importance of elucidating the impact of diversity in these systems.

Introduction

Wildlife disease dynamics are complex and influenced by numerous factors including host/pathogen identity (Gray et al. 2010, Gervasi et al. 2013, Johnson et al. 2013a, Shikano and Cory 2015), community structure and diversity (Johnson et al. 2008, Searle et al. 2011, Becker et al. 2014, Han et al. 2015, Young et al. 2017) and environmental conditions (e.g. Brearley et al. 2013, Hanlon and Parris 2014, Spitzen-van der Sluijs et al. 2014). Unravelling the dynamics of host-pathogen interactions is key to understanding and mitigating both emerging infectious diseases (EIDs) in wildlife and the potential for wildlife disease to impact humans (Daszak 2000, Belant and Deese 2010).

As animal populations and biodiversity decline globally (Chapin et al. 2000, Butchart et al. 2010), the relationship between disease and diversity has received increased attention (Johnson et al. 2013a, Halliday and Rohr 2019). At the forefront of these declines are amphibians, a group of special concern experiencing disease-related declines worldwide. (Blaustein 1994, Alford and Richards 1999, Stuart et al. 2004, Rohr et al. 2008, Blaustein et al. 2011). Multiple pathogens have been associated with amphibian population declines, including pathogenic fungi such as *Batrachochytrium dendrobatidis* (Bd) (Berger et al. 1998, Rohr et al. 2008) and *B. salamandrivorans* (Bsal; Health et al. 2018, Lötters et al. 2020) which cause the disease chytridiomycosis, Ranaviruses (Gray et al. 2009, Brunner et al. 2015), and trematode parasites (Szuroczki and Richardson 2009, Blaustein et al. 2011). These amphibian pathogens are globally distributed generalists, able to infect numerous amphibian species (Rohr, J.R. et al. 2009, Blaustein et al. 2018). Understanding the mechanisms underpinning host-pathogen relationships in amphibian systems is crucial for managing/informs global disease outcomes.

Research on amphibian population declines has led to an examination of relationships between amphibian host species and generalist pathogens using a variety of approaches (Blaustein et al. 2018). In natural systems more than one amphibian host and/or pathogen (Whitfield et al. 2013, Warne et al. 2016, Jenkinson et al. 2018) are present simultaneously, adding to the complexity of host-pathogen dynamics. With multiple potential host/pathogen species interacting, predicting disease outcomes is challenging. In multi-host systems which include a generalist pathogen, increased host diversity may reduce disease risk (Searle et al. 2011, Huang et al. 2013, Venesky et al. 2014, Civitello et al. 2015, Han et al. 2015), which in some systems has been identified as an effect of host species assembly order in a community (Johnson et al. 2013b). How multi-pathogen systems alter disease outcomes is currently less understood despite the commonality of coinfection in the wild (Hoverman et al. 2012, Stutz et al. 2018, Hoarau et al. 2020). Wuerthner et al. (2017) found positive effects on amphibian host survival to ranavirus infection when coinfected with trematode parasites, while Stutz et al. (2018) found the effect to be modulated by trematode species coinfecting. Other investigations have found context dependent effect of coinfection from multiple trematode species (Johnson and Hoverman 2012), additive effect of trematode-Bd coinfection (Romansic et al. 2011), effects of infection order and timing in Bd-ranavirus coinfection (Ramsay, C.T. and Rohr, J.R. 2020) and that coinfection by multiple chytrids reduced host survival (McDonald et al. 2020).

To determine the impacts of species diversity on host fitness we examined host-pathogen dynamics with three amphibian host species and three pathogen species. The western toad (*Anaxyrus boreas*) was selected as the focal host species due to reports of its declining populations and range (Muths et al. 2003, Davis and Gregory 2003, Wente et al. 2005, Slough and DeBruyn 2018) and because it is susceptible to numerous pathogens including ranaviruses

(Earl et al. 2016), trematode parasites (Johnson et al. 2001) and Bd (e.g. Blaustein et al. 2005, Carey et al. 2006, Han et al. 2008, Gervasi et al. 2013). Given evidence for a reduction of disease risk with increased amphibian host diversity (Searle et al. 2011, Han et al. 2015, Wuerthner et al. 2017) we hypothesized that increased host diversity would have a protective effect. Coinfection often has a negative effect on the host, however, in some contexts coinfection by trematodes has a positive effect on amphibian disease outcomes (Wuerthner et al. 2017). We expect coinfection with Bd and ranavirus will have deleterious effects on the hosts and that trematode coinfection effects will be context dependent.

Materials and Methods

Study system:

We experimentally examined three sympatric host and pathogen species found in the Oregon Cascades mountain range USA. The three amphibian host species are the Western toad (*Anaxyrus boreas*) our focal host species, the Pacific treefrog (*Pseudacris Regilla*) and the Cascades frog (*Rana Cascadae*). We challenged our host species with three pathogens, the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), the *iridiovirus* ranavirus (Rv) and *Echinostoma spp.* (Trem), the most prevalent trematode parasite in our study sites. Amphibian eggs were collected from naturally occurring breeding sites in Oregon in February and March of 2016. *P. regilla* were collected in the Willamette Valley at Dairy pond (44.572°N, -123.300°W and Parish Lake (44.522°N, -122.031°W). Eggs of *R. cascadae* were collected at Site 1 in Linn county (44.481°N, -121.994°W) while those of *A. boreas* were collected in the Deschutes National Forest (44.032°N, -121.687°W). We housed eggs in 40 L aquaria filled with dechlorinated water treated with AquaNova (Kordon LLC, Hayward CA, Item # 31161) and

Amquel (Kordon LLC, Hayward CA, Item # 31261). Within 48 hours of hatching, we moved larval amphibians into 40 L aquaria (treated as above) at densities of 1-2 animals per L and fed animals a mixture of rabbit chow, spirulina flakes, and shrimp flakes (3:1:1) ab libitum every other day. Complete water changes occurred weekly and temperatures remained between 12°C and 15°C.

Pathogen cultures:

We used the Newt Pond *Ranavirus* isolate collected in Iron River, Michigan (46.097°N, -88.644°W) from infected *R. sylvatica* tadpoles. The virus was cultured using a protocol adapted from Hoverman et al. (2010) wherein virus was passaged through fathead minnow cells incubated at 28°C without CO_2 and fed with Eagle's minimum essential medium with Hank's salts (HMEM) and 5% fetal bovine serum. The virus was stored at -80°C until the start of the experiments. For the virus treatment, 10^5 plaque forming units (PFU) were added to 10L, for a concentration of 10^2 PFU per mL. These concentrations mirror those we've used our prior ranavirus study (Snyder et al. 2020b).

Bd was cultured from the JEL-646 isolate (isolate from *P. regilla* in Point Reyes, California), obtained from Joyce E. Longcore, University of Maine. Isolates were grown first in a 1% tryptone broth until growth was visible. One mL of the Bd-tryptone broth was plated onto 100mm culture dishes containing 1% tryptone agar 1 to 2 weeks prior to experimentation. For experimentation, Bd zoospores were washed off agar plates with a rubber spatula and 10mL of water, were pooled and quantified via hemocytometer at 400X. Zoospore solution was added to 10L aquaria for a final concentration of 10^3 zoospores/mL, the same concentration used in our prior Bd study (Snyder et al. 2020a).

For trematode collection, Ramshorn snails were collected from William L Finley National Wildlife Refuge (44.427°N, -123.312°W). Snails were placed in 6-well plates with water and spinach and warmed under a heating lamp for one-hour to encourage shedding of trematode parasites prior to screening via dissecting microscope. Infected animals were marked and their parasites identified at 400X via a compound light microscope. *Echinostoma spp*. were the most common trematode infection. Snails shedding *Echinostoma spp*. and only *Echinostoma spp*., were housed in three 10L aquariums in an incubator at 70°C. The incubator remained on the night cycle (no lights) to avoid excessive reinfection of the host snails. At time of experimental infection, snails are placed in 6-well for shedding as above. Cercariae were then collected via disposable glass pipette and deposited in a 0.5L glass container for use within the hour.

Experiment:

To examine effects of ecologically relevant single- and concurrent- pathogen exposures at two levels of host richness, we designed a 14-day experiment, in which a naïve population of one or three host species were exposed to one or more pathogen exposed conspecifics and/or trematode cercariae. We also conduct truncated 2- and 7-day experimental replicates, to compare temporal effects of exposure.

To examine potential effects of coinfection with the three pathogens on amphibian hosts, we used a factorial design with eight exposure treatments, three duration treatments, and two diversity treatments (Fig. 1). For each treatment combination, two replicate 10L aquaria each contained nine unexposed animals and one Bd or sham exposed *A. boreas* and one Ranavirus exposed or sham *A. boreas*, by treatment. For 1-host treatments all animals are *A. boreas*,

whereas in 3-host treatments the nine naïve animals include three of each *A. boreas*, *P. regilla* and *R. cascadae*.

One week prior to experimentation, a pool of animals exposed to Bd/sham and Rv/sham were tagged with a visible implant elastomer (VIE) under the skin, laterally near the base of the tail. Bd treatment animals were tagged with an orange VIE and Rv treatment animals were tagged with a green VIE. These tags allowed us to distinguish the naïve animals from the pathogen exposed animals added later.

Three days prior to experiment start, naïve animals were moved to 40L aquaria by species at densities of 50 to 80 animals per aquaria. These animals were chosen haphazardly from our laboratory populations. Exposure treatments were prepared two days prior to the experiment being conducted. For the Bd exposure treatment, animals were moved into 10L aquaria into which either Bd inoculate (as described above) or a sham inoculate was added. Similarly, ranavirus exposure animals were moved into 10L aquaria into which either ranavirus inoculate (as described above) or a sham inoculate was added. One day prior to the experiment, naïve animals were moved into their experimental units (10L aquaria).

At the start of the experiment, Bd/sham exposed and Rv/sham exposed animals were added to aquaria by treatment. Trematode cerariae were collected in a solution as described above and divided into 24x 50ml Falcon tubes and added to appropriate treatments. This trematode procedure was repeated on day 7 for animals continuing to day 14. For the duration of the experiment animals were fed *ad libidum* and each 10L aquaria was fitted with an oxygen bubbler. At experimental endpoints, animals were euthanized in MS-222, measurements recorded and then animals were frozen.

Analysis:

For both 1- and 3- host diversity treatments, the effects of exposure treatment on mortality was examined with an ANOVA and treatments were compared against controls using Dunnett's multiple comparisons test (Dunnett 1955).

For both 1- and 3- host diversity treatments the effects of exposure treatment, treatment duration and their interaction on avg daily change in SVL was compared via a 2-way ANOVA, and Dunnett's multiple comparisons were used to compare treatments against the control. Within exposure treatments, mean daily change in SVL was compared via Tukey's multiple comparisons test. For both 1- and 3- host diversity treatments, the effect of exposure treatment on change in Gosner (1960) developmental stage was compared via a Brown-Forsythe one-way ANOVA (Glantz and Slinker 2001), and treatments were compared against controls via Dunnet's T3 multiple comparisons test (Dunnett 1980).

Results

For analyses and results the following treatments are abbreviated as follows: *Batrachochytrium dendrobatidis* (Bd), Ranavirus (Rv), and trematode (Trem). For coinfection treatments abbreviations are joined with a plus (+). For example: The treatment exposed to Bd, ranavirus, and trematodes is written as Bd + Rv + Trem.

In the 1-host diversity treatments, mean mortality was influenced by exposure treatment (ANOVA $F_{7,8}$ =6.0, P=0.0109), with the Bd + Trem treatment significantly different from the control (Dunnett's, P=0.0059; Fig. 2.). No difference between mean mortality was detected in 3-host treatments (ANOVA $F_{7,8}$ =2.286, P=0.1348)

In our 1-host treatments, for mean daily change in SVL of *A. boreas* there was a significant interaction between exposure treatment and treatment duration (ANOVA $F_{14,401}$ = 10.21, $P < 0.0001$). In the 3-host treatments *A. boreas* had a significant interaction between exposure treatment and treatment duration for mean daily change in SVL (ANOVA $F_{14,117}$ = 4.34, $P < 0.0001$), but not *R. cascadae* (ANOVA $F_{14,115} = 0.45$, $P = 0.9526$) or *P. regilla* (ANOVA $F_{14,117} = 1.43$, $P = 0.1491$).

In 1-host *A. boreas* diversity treatments, Bd + Rv (Dunnett's, $P<0.0001$), Rv + Trem (Dunnett's, $P<0.0001$) and Bd + Rv + Trem (Dunnett's, $P<0.0001$) exposure treatments differed significantly in daily SVL change from control at day two. In 3-host *A. boreas* diversity treatments, Bd + Rv (Dunnett's, $P=0.019$), Bd + Trem (Dunnett's, $P=0.0007$) and Bd + Rv + Trem (Dunnett's, $P=0.0021$) were significantly different from controls at day two (Fig. 3).

Within exposure treatments mean daily change in SVL of *A. boreas* was significantly different between days 2 and 7 in 1-host Bd (Tukey's, $P=0.0002$), 1-host Trem (Tukey's, $P<0.0001$), 1-host Bd + Rv (Tukey's, $P=0.007$), 1-host Bd + Trem (Tukey's, $P=0.0077$), 1-host Rv + Trem (Tukey's, $P=0.0004$), 3-host Bd + Trem (Tukey's, $P<0.0001$) and 3-host Bd + Rv + Trem (Tukey's, $P=0.0133$) treatments. Within treatments mean daily change in SVL in *A. boreas* was significantly different between days 2 and 14 in 1-host Control (Tukey's, $P=0.0435$), 1-host Bd (Tukey's, $P<0.0001$), 1-host Trem (Tukey's, $P<0.0001$), 1-host Bd + Rv (Tukey's, $P=0.0004$), 1-host Bd + Trem (Tukey's, $P=0.0288$), 1-host Rv + Trem (Tukey's, $P<0.0001$) and 3-host Bd + Trem (Tukey's, $P<0.0001$; Fig. 2). For all *R. cascadae* (3-host) treatments mean daily SVL change did not differ from controls and within treatments the avg daily SVL change did not differ by treatment duration. For *P. regilla* (3-host) treatments mean daily SVL change did not differ from controls. Within exposure treatments, mean daily SVL change for *P. regilla*

was significantly different between days 2 and 14 for the Bd treatment (Tukey's, P=0.002; Fig. 3).

In the 1-host diversity treatments exposure, treatment had a significant effect on development (Brown-Forsythe ANOVA $F_{7,113.8}$ =10.59, P<0.0001). Three exposure treatments were different from the control: Trem (Dunnet's T3, P=0.0022), Bd + Rv (Dunnet's T3, P<0.0001), and Rv + Trem (Dunnet's T3, P=0.0093; Fig. 3). On average *A. boreas* developmental stages progressed from an average Gosner stage of 26 (Std Dev 0.514) initial to an average stage of 26.35 (Std Dev 0.828) at day 14.

In the 3-host diversity treatment, exposure treatment had a significant effect on change in Gosner stage for *A. boreas* (Brown-Forsythe ANOVA $F_{7,24.51}$ =3.537, P=0.0092), but not for *R. cascadae* (Brown-Forsythe ANOVA $F_{7,17.62}$ =2.348, P=0.0694) or *P. regilla*. (Brown-Forsythe ANOVA $F_{7,15.66}$ =1.397, P=0.2740) For 3-host *A. boreas* the Bd + Trem exposure treatment differed from control (Dunnet's T3, P=0.0477; Fig. 4). In 3-host treatments, *A. boreas* Gosner stages went from an average stage 25.98 (Std Dev 0.398) initial to an average stage of 26.350 (Std Dev 0.828) on day 14. *R. cascadae* developmental stages progressed from an average stage 27.09 (Std Dev 1.571) initial to stage 27.98 (Std Dev 2.198) on day 14, while *P. regilla* grew from an average stage of 27.04 (Std Dev 1.906) initial to an average stage of 27.89 (Std Dev 2.424) on day 14.

Discussion

Mortality occurred during our 14-day experiment and the mortality in our 1-host Bd + Trem treatment differed from controls. While Bd mortality often occurs over a duration greater than two weeks (Berger et al. 2005, Retallick and Miera 2007, Voyles et al. 2017), other studies

have shown mortality in *A. boreas* from Bd exposure within 48 hours (Blaustein et al. 2005) and that Bd can produce chemicals that cause pathology in the absence of infection (McMahon et al. 2013). Chytridiomycosis outcomes are also effected by the presence of addition stressors (Parris and Cornelius 2004, Searle et al. 2010, Hanlon and Parris 2014). Given that the presence of both Bd and trematodes resulted in increased mortality suggests an additive or synergistic effect between these pathogens and is not without precedent as previous efforts that found some trematode coinfection beneficial (Wuerthner et al. 2017). However, coinfections by *Echinostoma spp.* used here were negatively associated with presence of Bd in the field (Stutz et al. 2018). The increased mortality in this coinfecting pair may explain such negative correlations in the wild.

We found several differences in mean daily change in SVL (proxy for growth), both between and within exposure treatments. In the 1-host density-, 2-day duration- treatments, average daily change in SVL for *A. boreas* is different from controls in three exposure treatments; the Trem treatment displaying faster growth and Bd + Rv, and Bd + Rv + Trem treatments with depressed growth as compared to controls (Fig. 3). This initial increase in growth rate could explain the beneficial effect of trematodes demonstrated by Wuerthner et al. (2017) as increased host mass may increase tolerance (Garner et al. 2009, Kilpatrick et al. 2010, Johnson et al. 2011).

In our 3-host density-, 2-day duration- treatments, average daily change in SVL for *A. boreas* also differed from controls in three treatments; the Bd + Trem treatment with faster growth and Bd + Rv, and Bd + Rv + Trem treatments depressed growth as compared to controls (Fig. 3). Within exposure treatments, including 1-host controls, mean daily change in SVL in the 2-day duration treatments are different from the daily change in SVL in one or both of the 7- and

14- day treatments (Fig. 3). There were no significant differences between or within exposure treatments for *R. cascadae* or *P. regilla*.

Daily change in SVL was most varied in the first two days of the experiment. The move to the experimental aquaria and addition of pathogen exposed conspecifics, and trematodes, could stimulate a change in feeding/growth rates. In the three-host treatments the addition of amphibian species likely also plays a role in changes to feeding/growth rates. In both 1- and 3-host diversity treatments the Bd + Rv and Bd + Rv + Trem treatments saw reduced daily change in SVL compared to controls. Given the relatively short period of exposure for the 2-day duration treatment, it is likely that the change in growth rate is a response to the presence of infected conspecifics. We cannot, however, determine if this is a response to the presence of conspecifics with two different pathogens, a response to an increased percentage of infected conspecifics (1/11 vs 2/11), or a combination of both. For our 1-host Trem and 3-host Bd + Trem treatments, which experienced increase growth rates in the first 2 days, the presence of trematode cercariae are a commonality with the response appearing to be modulated by host diversity. Previous examinations of effects of *Echinostoma spp*. exposure on amphibian hosts have demonstrated changes in activity (Preston et al. 2014, Reynolds and Reynolds 2017) and growth (Koprivnikar et al. 2008).

We also found developmental differences when comparing the mean number of developmental stages over the 14-day experiment. Like growth, amphibian development responds to a wide range of biotic and abiotic factors (Werner 1986, Newman 1992, Koprivnikar et al. 2008, Haislip et al. 2011), so differences between exposure- and diversity- treatments were expected. Development was reduced compared to control in four treatments, 1-host Trem, Bd + Rv, Rv + Trem and 3-host Bd + Trem (Fig. 4). While development was depressed in both 1-host

Trem and 1-host Bd + Rv, these two treatments experienced opposite changes in mean daily change in SVL in the first two days (Fig. 3).

We have shown that wildlife disease outcomes may be influenced by factors such as the number of host species present, the identity of those species and the interactions between them, as well as the number of- and identity of- pathogens present. We found that *A. boreas* daily growth-rate at the start of the experiment was not equal to the mean growth rates over longer durations and that the direction of the change was determined by treatment. Future work could examine any acute responses of exposure over a shorter fine scale timeline, or examine how transmission of these pathogens is altered by comparing infection prevalence through time.

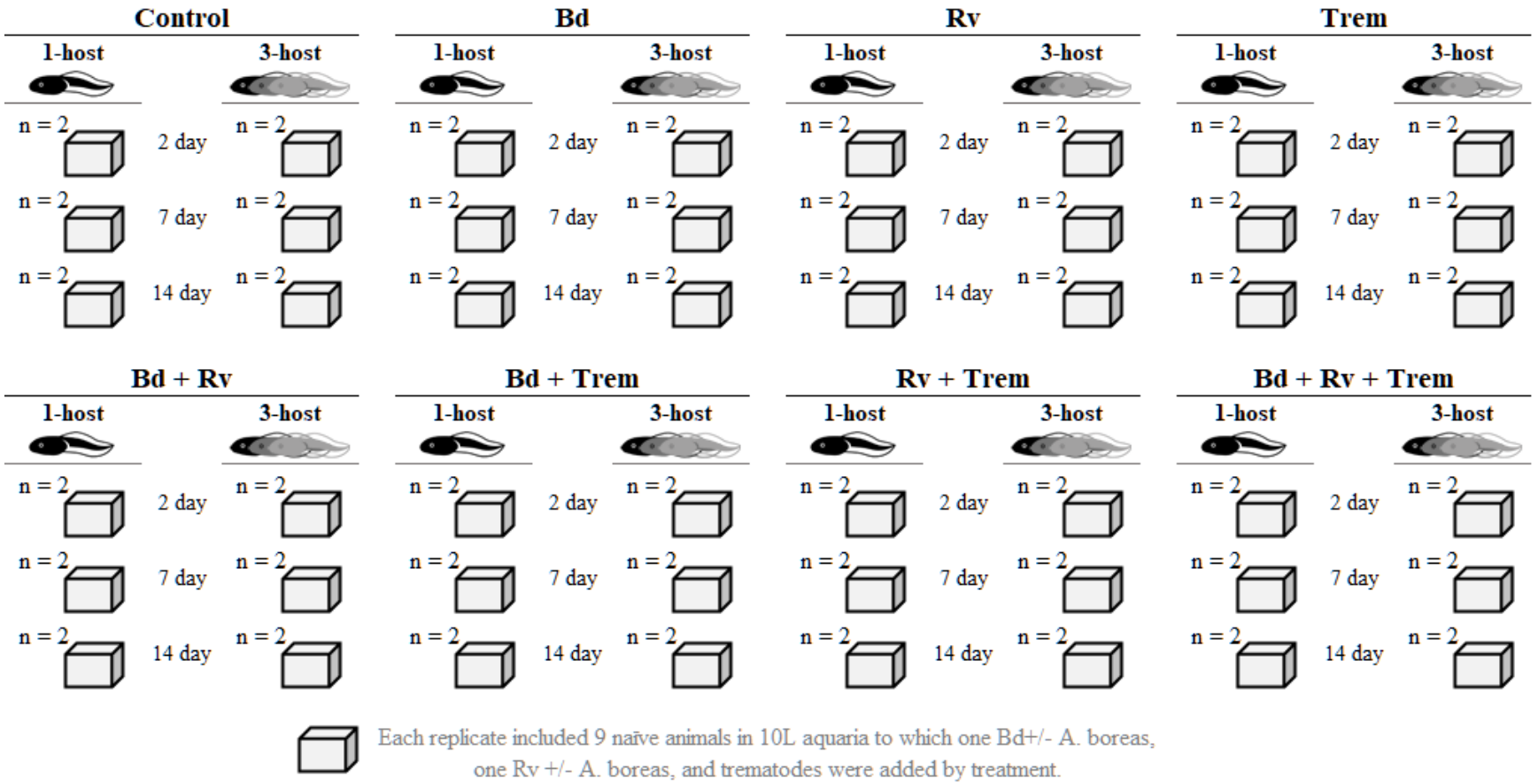

Fig 1. Graphic representation of the experiment's 48 treatments, including eight exposure treatments, three duration treatments and two levels of diversity.

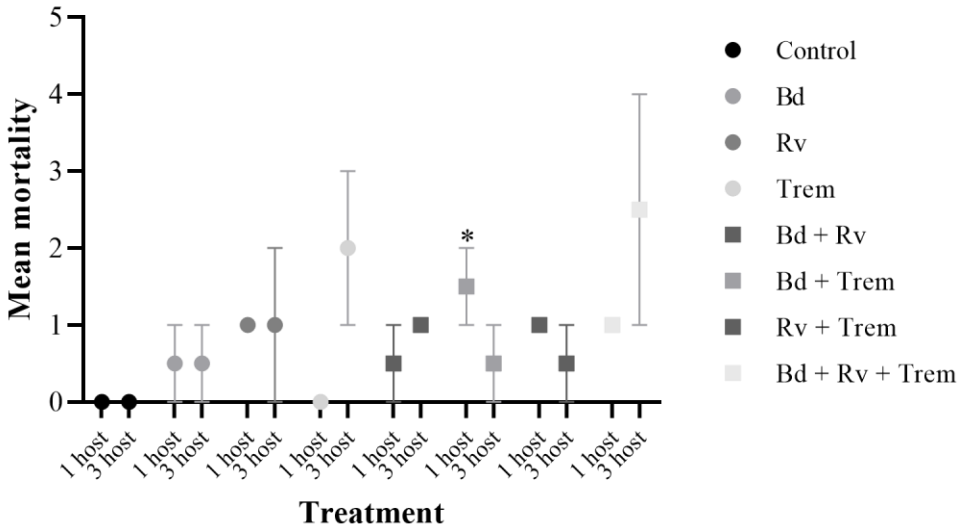

Fig 2. Average number of deaths (of 11 animals) by treatment and diversity treatment at the end of the experiment (day 14). * treatment which is significantly different from the control.

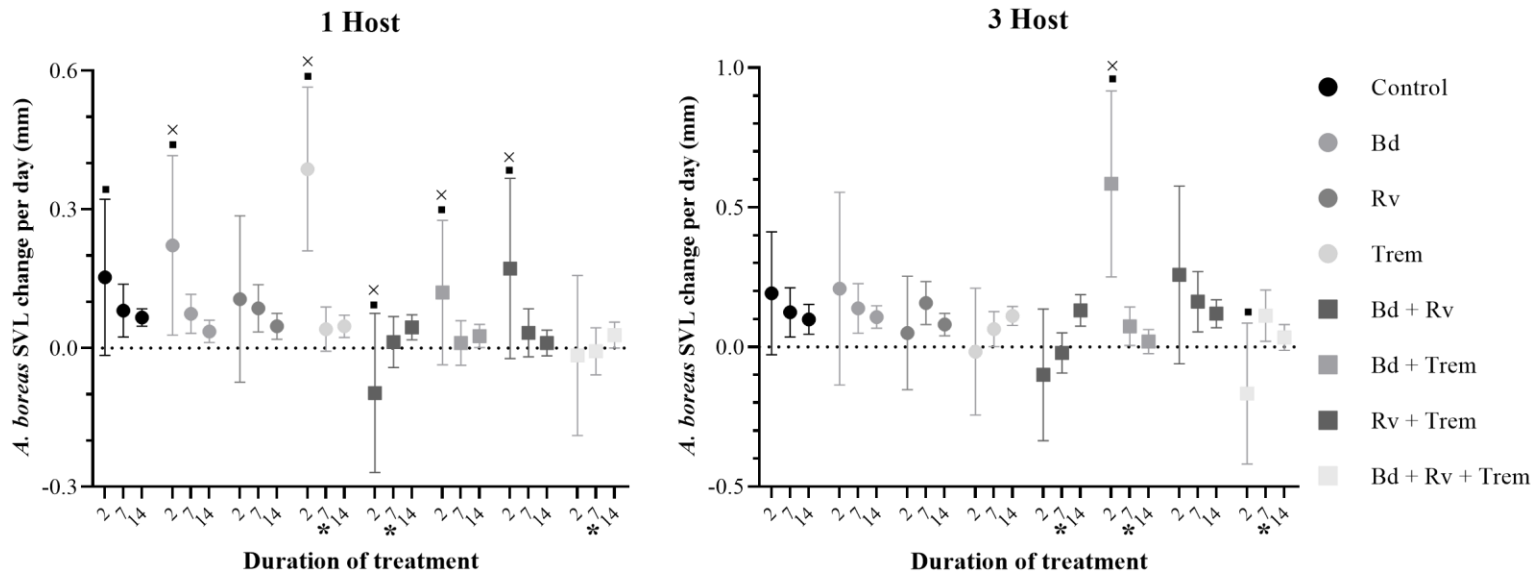

Fig 3. Average daily change in snout-vent length (SVL) of *A. boreas* in 1- and 3- host treatments. Within treatments, (x) denotes day 2 and 7 are significantly different and (■) denotes day and 14 are significantly different. * treatments which differed significantly from control at day two.

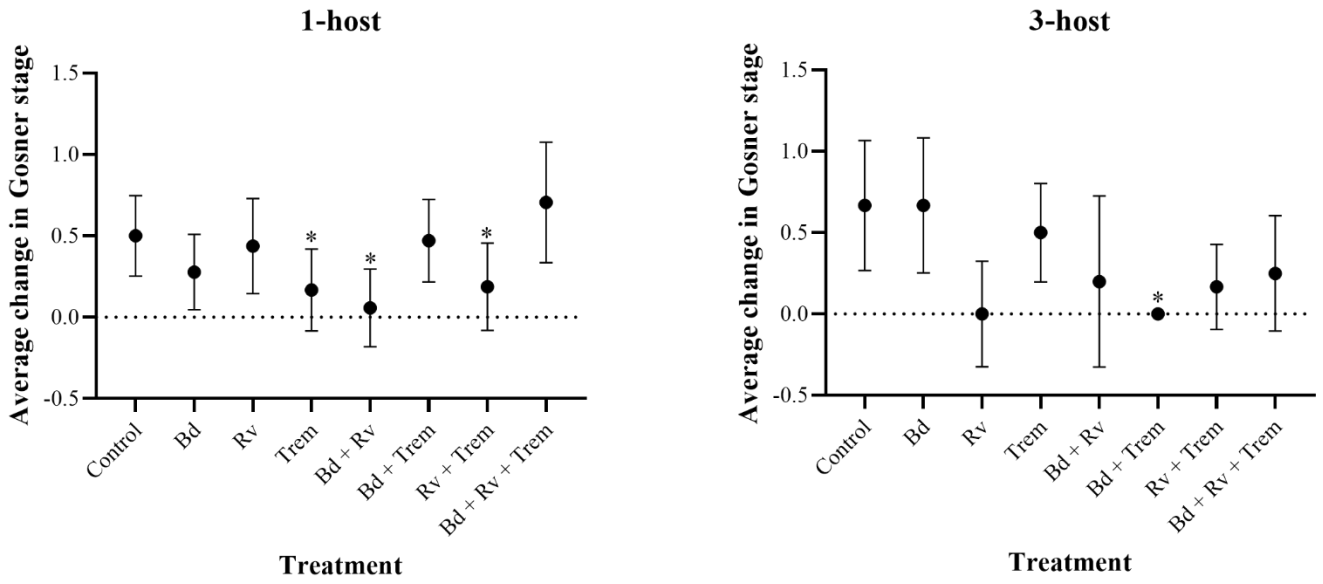

Fig 4. Average change in Gosner (1960) developmental stage of A. boreas over the 14-day experiment. *treatments which differed significantly from controls.

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CHAPTER 5 – CONCLUSION

Paul W. Snyder

Conclusion

Amphibians are host to multiple globally distributed emerging infectious diseases including the fungal pathogens *Batrachochytrium dendrobatidis* (Bd) (Berger et al. 1998, 1999, Fisher et al. 2009, Rödder et al. 2009, Olson et al. 2013, Scheele et al. 2019, Lambert et al. 2020, Fisher and Garner 2020) and *Batrachochytrium salamandrivorans* (Bsal) (Grant et al. 2016, Stegen et al. 2017, Yap et al. 2017, Health et al. 2018) as well as a dsDNA virus, ranavirus (Chinchar 2002, Greer et al. 2005, Robert 2010, Price et al. 2014, Duffus et al. 2015). Further impacting amphibian population declines are trematode parasites which utilize the amphibians as a second intermediate host, especially those of the family *Echinostomatidae* and genus *Ribeiroia* which may facilitate population level effects via reduced growth, malformed limbs, or direct mortality (Fried et al. 1997, Blaustein and Johnson 2003, Rohr, J.R. et al. 2009).

Amphibian disease-mediated population declines and extinctions in the ongoing biodiversity crisis have drawn special attention to the relationships between disease and diversity. Changes in host diversity and community composition can alter disease prevalence (e.g. Ostfeld and Keesing 2000, Begon 2008, Civitello et al. 2015, Huang et al. 2016, Young et al. 2017) and understanding the relevant factors involved in this relationship will be key for amphibian conservation efforts and for grappling with the ongoing biodiversity crisis.

In this dissertation, using an experimental approach, I examined the role of diversity in disease dynamics in amphibians. In chapter two I explored the effects of host diversity on ranavirus disease dynamics. Using laboratory and mesocosm

experiments I found that community composition altered ranavirus disease dynamics. The laboratory experiment provided a baseline for comparison of the effects of ranavirus on the host species. In the laboratory, via direct exposure, I determined that one of the host species, the Pacific tree frog (*Pseudacris regilla*), was susceptible to ranavirus while survival in other hosts, the Western toad (*Anaxyrus boreas*) and the Cascades frog (*Rana cascadae*) was not different from unexposed hosts in control regimes. In the mesocosm experiment ranavirus exposed conspecifics were introduced into naïve populations of either one-host (*A. boreas*) or three-hosts (*A. boreas*, *R. cascadae* & *P. regilla*) and monitored for 60 days. At day 30, mortality in ranavirus exposed one-host treatments was not different from one-host control treatments, while ranavirus exposed three-host treatments experienced increased mortality. At the end of the experiment (60 days), survival was reduced in ranavirus exposed one-host treatments compared to one-host controls while three-host treatments were further reduced, experiencing nearly 100% mortality. *A. boreas* which was not susceptible to ranavirus in the laboratory experiment, experienced increased mortality in the outdoor mesocosms when in the presence of the highly susceptible *P. regilla*. Our results also varied between the controlled laboratory experiment - where *A. boreas* survival was unaltered by exposure to ranavirus – and our outdoor mesocosms, where survival of ranavirus exposed *A. boreas* was reduced compared to controls. These results highlight the need for pairing laboratory and larger scale examinations in such complex systems.

In chapter three I conducted a mesocosm experiment similar to that described in Chapter two with *Batrachochytrium dendrobatidis*. I found animals exposed to Bd

in one-host treatments had reduced survival compared with those in unexposed control treatments. Survival of hosts in Bd exposed three-host mesocosms was not different from hosts control groups offering further support that increased host diversity reduces Bd disease risk.

In chapter four I expanded our investigation of diversity and disease to include pathogen diversity. Here I utilized a laboratory experiment to explore the effects of single- and concurrent- infections with Bd, ranavirus and trematode parasites (*Echinostoma spp.*) at two levels of host diversity. Animals in one-host treatments coinfected with Bd and trematodes had reduced survival compared to controls, while in three-host treatments Bd-trematode coinfection did not reduce survival. I also found that growth rate in hosts during the first two days of exposure to infected conspecifics was variable and treatment dependent. Outcomes were altered by both host and pathogen diversity demonstrating a wide range of effects on disease as diversity of host or pathogen is altered. The range of responses in this laboratory experiment highlight the complexity of disease dynamics in amphibian systems and the importance of considering host- pathogen diversity and the full scope of host/pathogen interactions in a given system.

In summary I have demonstrated that changes in diversity affect disease outcomes and that host and pathogen identity influence the direction and scale of such effects. I have provided further evidence that increased host diversity may reduce the risks of Bd in a community. The same changes to host diversity in a ranavirus exposed community could erase amphibian populations. To understand disease dynamics of generalist pathogens and their hosts, we must consider the full range of

interacting species in a community. Appreciating the inherent complexity of wildlife disease dynamics is requisite for any conservation efforts aimed at species, such as amphibians, experiencing disease mediated population declines. More generally, I have demonstrated that pairing laboratory and field experiments is necessary in the study of wildlife disease, as context matters.

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