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Evaluation of Treatment Options and Investigation of Pathogenesis of Chytridiomycosis in North American Salamanders

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To the Graduate Council:

I am submitting herewith a dissertation written by Anastasia Elaine Towe entitled "Evaluation of Treatment Options and Investigation of Pathogenesis of Chytridiomycosis in North American Salamanders." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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Evaluation of Treatment Options and Investigation of Pathogenesis of Chytridiomycosis in North American Salamanders

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Anastasia Elaine Towe

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Dedication

I dedicate this work to my little brother Justin, who encouraged me to chase my dreams.

I miss you more than words can say.

Don't pause too long.

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Abstract

Chytridiomycosis, a cutaneous fungal disease caused by two related fungi, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*), is a major cause of amphibian population declines worldwide. *Bd* has a broad distribution, covering every continent except for Antarctica. Meanwhile, *Bsal* is currently restricted to Europe and Asia. Treatment options for both *Bd* and *Bsal* remain a major area of concern for wild populations. Recently, implants for long-term, parenteral antifungal drug administration have shown some promise in fungal diseases in wildlife. We investigated the safety and efficacy of an intracoelomic implant of the antifungal drug terbinafine in greater sirens exposed to *Bd*. While the efficacy results were inconclusive, the implants did not cause harm and led to detectable levels of terbinafine in plasma. The use of such implants in amphibians remains an important area for further investigation. In a separate study, we explored the use of a probiotic bacteria and addition of a known secondary bacterial pathogen in *Bsal*-infected animals. The objectives of this study were to determine the safety and efficacy of using probiotic bacteria to prevent and treat *Bsal* chytridiomycosis, and to evaluate bacteremia and septicemia as components of morbidity and mortality in animals that succumb to disease. While the probiotic treatment did not impact mortality rates, there was evidence of bacterial infiltration into the blood and organs in *Bsal*-exposed animals. Finally, while there is an ever-growing body of research elucidating the acute impacts of *Bsal*, sublethal effects on population dynamics must be considered as well. We used a two-part study design to investigate the impacts of *Bsal* exposure on individual reproductive potential, and on reproductive success under breeding conditions. This study provided evidence for terminal investment, increased reproductive effort in the face of pathogen exposure, in eastern newts exposed to *Bsal*. Overall, the findings of this research provide more information about new avenues for treatment for chytridiomycosis and their potential drawbacks, and expand on the existing knowledge of *Bsal* pathogenesis. These findings will provide a foundation for future amphibian disease research, informing mitigation efforts for this disease in managed and free-ranging amphibian populations.

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Introduction

Numerous wildlife are currently in decline, and amphibians are among the most threatened, with nearly 60% of species in danger of extinction over the last 40 years (García-Rodríguez et al., 2022). There are many contributors to amphibian population declines, including habitat loss, environmental toxins, and infectious disease. One such disease is chytridiomycosis, a cutaneous fungal disease caused by two related fungi, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*). Chytridiomycosis has led to an amphibian panzootic that represents the greatest recorded disease-related loss of biodiversity, with *Bd* contributing to population declines in over 500 amphibian species (Scheele et al., 2019). While *Bsal*'s population impacts are currently more limited in scale, the risk of exposure to naïve, susceptible species is of great concern.

Bd has a broad distribution worldwide, with over 1000 confirmed host species in 86 countries, covering every continent except for Antarctica. (Castro Monzon et al., 2020). Meanwhile, *Bsal* is not yet known to occur in North America, with its current distribution restricted to Europe and Asia (Lötters et al., 2020; Waddle et al., 2020). In Western Europe, *Bsal* has contributed to the near extirpation of fire salamander (*Salamandra salamandra*) populations (Martel et al., 2013). *Bd*'s emergence as a global pathogen has been linked to the expansion of the commercial trade in amphibians (O'Hanlon et al., 2018), and there is a high likelihood that *Bsal* could be spread the same way. *Bsal* has been detected in the pet trade, and there are concerns that this could be the introduction point for the pathogen to currently naïve populations (Nguyen et al., 2017). With continued global movement of amphibians, *Bsal* poses a significant threat to North American amphibian biodiversity, and many species have been shown to be susceptible through laboratory trials (Carter et al., 2020; Friday et al., 2020; North American Bsal Task Force, 2020).

Whether caused by *Bd* or *Bsal*, chytridiomycosis disrupts the integrity of the skin. Immature sporangia infect cells in the superficial epidermis a few layers deep, maturing at the same rate that the host cells move outwards as they mature and keratinize. As the host cell dies, the sporangia develop discharge tubes that open to the surface of the skin (Berger, Hyatt, et al., 2005,

Martel; et al., 2013). In *Bd* infections, hyperkeratosis is caused by an increase in epidermal cell turnover and the swelling of epidermal cells near the site of infection along with sporangia causing premature death and keratinization Berger, Hyatt, et al., 2005). Meanwhile, *Bsal* infections tend to cause more rapid destruction of keratinocytes as the zoospores encyst on the skin, causing erosive lesions that develop into deep ulcers (Martel et al., 2013) .The disruption of normal cell architecture leads to systemic disease through detrimental effects on hydration, ion exchange, and cutaneous respiration. Excessive water loss leads to dehydration and electrolyte imbalances, and in *Bd* this has been shown to lead to cardiac arrest and death (Salla et al., 2018; Voyles et al., 2009; Wu et al., 2019). While similar studies on *Bsal* pathogenesis are still underway (Sheley et al., 2023), it is expected that the deep ulceration and necrosis of the skin caused by the pathogen has similar effects on electrolyte balance. It has also been shown that this skin erosion and ulceration promotes secondary bacterial overgrowth that may result in lethal septicemia (Bletz et al., 2018).

Treatment options for both *Bd* and *Bsal* remain a major area of concern, particularly for wild populations. Various topical treatment options have been explored for both fungi, but the necessity of repeat dosing for individual animals makes many of these regimens impractical for free-ranging animals. The use of chemical antifungals in natural environments is also concerning, as there may be unintended negative consequences for other organisms in the community. Recently, implants for long-term, parenteral antifungal drug administration have shown some promise in other fungal diseases in wildlife (Hardman et al., 2021; Kane et al., 2017). In Chapter I, we investigate the safety and efficacy of an intracoelomic implant of the antifungal drug terbinafine in greater sirens exposed to *Bd*.

Probiotics are another treatment avenue that has been explored in various disease processes, including fungal diseases in wildlife. Under laboratory conditions, the use of probiotic bacteria to augment the natural microbiome has led to decreased *Bd* load and increased survival rates in frogs (Harris et al., 2009). However, the ulcerative effect of *Bsal* on the skin barrier creates a potential pathway for secondary bacterial infiltration, raising the concern that probiotic treatment may in fact be detrimental in the face of existing *Bsal* lesions and a weakened immune system. In Chapter II we explore both the use of a probiotic bacteria and addition of a known secondary

bacterial pathogen in *Bsal*-infected animals. The objectives of this study were to determine the safety and efficacy of using probiotic bacteria to prevent and treat *Bsal* chytridiomycosis, and to evaluate bacteremia and septicemia as components of morbidity and mortality in animals that succumb to disease.

Finally, while there is an ever-growing body of research elucidating the individual, acute impacts of *Bsal*, adult morbidity and mortality are only part of the story when it comes to predicting population declines; sublethal effects on recruitment of the next generation must be considered as well. In the case of *Bd,* previous studies have investigated these effects, including gross and histologic changes to gonads in infected animals (Brannelly et al., 2021), long-term impacts on body fat and gonad size in susceptible species (Campbell et al., 2019), and impacts on mating behaviors even after the infection has been cleared (An & Waldman, 2016). Field studies on *Bd* have also been incorporated into population level modeling to explain the extinction of some and persistence of other populations in the face of chytridiomycosis outbreaks (Briggs et al., 2005). This is an area that has not previously been explored in *Bsal* pathophysiology and epidemiology. In Chapter III, we use a two-part study design to investigate the impacts of *Bsal* exposure on individual reproductive potential, and on reproductive success under breeding conditions.

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Chapter I: Evaluation of intracoelomic implants for terbinafine administration for prevention of chytridiomycosis in greater sirens (*Siren lacertina*)

Abstract

Chytridiomycosis caused by *Batrachochytrium dendrobatidis* (*Bd*) has been documented in greater sirens (*Siren lacertina*) in the wild and in the pet trade. This study evaluated the use of terbinafine-impregnated implants, in prevention of *Bd* infection in greater sirens. Implants were placed intracoelomically in both control (blank implant, n=4) and treatment (terbinafine implant, n=4) groups. Sirens were exposed to *Bd* zoospores at one- and two-months post-implant placement. Blood was collected monthly for plasma terbinafine levels, and skin swabs were collected weekly for *Bd* qPCR. Animals with terbinafine implants had detectable concentrations of plasma terbinafine ranging from 17-102 ng/mL. Only one animal had a peak concentration above the published minimum inhibitory concentration for terbinafine against *Bd* zoospores (63 ng/mL). Terbinafine implants did not prevent infection or affect *Bd* clearance rate, but no adverse effects from implants were observed. These findings indicate using intracoelomic drug implants for drug delivery in amphibians is safe; however, terbinafine efficacy in preventing *Bd* infections in sirens remains unclear. Further investigation of the use of intracoelomic implants and identification of effective drugs and doses in other amphibian species is warranted, as this may provide a practical method for long-term drug delivery in wildlife.

Introduction

Batrachochytrium dendrobatidis (*Bd*) is a fungal pathogen that causes severe, often lethal disease in amphibians (Scheele et al., 2019). *Bd* infects keratinized skin, leading to hyperkeratosis, epidermal hyperplasia, disorganization of the epidermal cell layers, and skin sloughing (Berger, Speare, et al., 2005)(Berger, Speare, et al., 2005). This impacts water uptake and ion exchange in affected animals, and death is thought to occur due to asystolic cardiac arrest caused by severe electrolyte imbalances (Voyles et al., 2009). Chytridiomycosis caused by *Bd* has led to the decline of over 500 amphibian species worldwide and 90 presumed extinctions (Scheele et al., 2019).

The greater siren (*Siren lacertina*) is a large, fully aquatic salamander that can be found in the Atlantic and Gulf coastal plains of the southern and eastern United States (Hendricks, 2005)(Hendricks, 2005). It is categorized by the International Union for Conservation of Nature as Least Concern and is common throughout the central regions of its range. However, population status is variable and even rare in the peripheral regions of the range, and in Maryland it is considered state endangered (IUCN SSC Amphibian Specialist Group, 2022). The aquatic lifestyle of sirens makes their populations difficult to monitor (Hendricks, 2005). Previous studies have found *Bd*-infected greater sirens in the pet trade (Tamukai et al., 2014) and both greater and lesser sirens (*Siren intermedia*) in the wild (Chatfield et al., 2012; Talley et al., 2011), but the overall population impacts of *Bd* are unknown.

Application of topical antifungals is effective at reducing *Bd* loads, however treatment courses tend to require multiple dosing events and fail to prevent infection long-term in some cases (Geiger et al., 2017). Therefore, topical treatments are most practical in managed care settings where animals can be handled daily and maintained in pathogen-free environments, and less practical in the wild. Terbinafine is a lipophilic antifungal agent that is commonly used to treat superficial fungal infections(Newland & Abdel-Rahman, 2009). It has been used topically in the treatment of chytridiomycosis with no reports of toxicity, but as with other topical treatments, repeated dosing has been necessary to clear infection (Guzman et al., 2022), and clearance has not been 100% successful (Roberts et al., 2019). Subcutaneous implants

impregnated with terbinafine have previously been used in pharmacokinetic studies in cottonmouths (Kane et al., 2017) and hellbenders (Hardman et al., 2021), with the goal of using them for treatment of snake fungal disease and chytridiomycosis, respectively. These implants are produced commercially for melatonin administration in dogs and ferrets(Murray, 2005; Verschuuren et al., 2022) and are approximately the size of typical Passive Integrated Transponders (PIT) tags, or a grain of rice. Subcutaneous terbinafine implants in hellbenders provided plasma concentrations at or above the minimum inhibitory concentration (MIC) for *Bd* zoospores for 2 to 15 weeks consecutively (Hardman et al., 2021). An attempt to place the implants subcutaneously in sirens failed, as the implants were rejected and expulsed (unpublished study). Therefore, the route was changed to intracoelomic for this study.

The objectives of this study were to 1) evaluate the safety of intracoelomic terbinafine implant placement, 2) quantify blood levels of terbinafine in intracoelomic implanted animals, and 3) evaluate the efficacy of terbinafine delivered through an intracoelomic implant in preventing *Bd* infection.

Methods

Animal Intake and Husbandry

This study was conducted at the University of Tennessee, Knoxville and all procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee Protocol no. 2723. Greater Sirens (n=9) were collected from the wild and skin swabs were performed for *Bd* testing via PCR. Animals were handled with nets and moistened towels and were swabbed five times each on the ventrum, head, and feet(Chatfield et al., 2012; Clare et al., n.d.). Swabs were placed in sterile microcentrifuge tubes and stored at -80C. A standard *Bd* qPCR protocol was followed as in Boyle et al (2004). Reactions were run in duplicate; positive controls and standard curves were constructed using serial dilutions of cultured *Bd* zoospores, and samples with a CT below 40 were considered positive. Animals initially tested positive for *Bd* at low levels. Fungal cultures were performed on the swab samples and an isolate from one animal was submitted to the Rosenblum Lab at UC Berkley for sequencing, which identified the isolate as Bd-GPL1. The sirens were heat-treated to clear *Bd* infections approximately 16 months

prior to the start of this study and confirmed negative via *Bd* PCR performed on two consecutive skin swabs. Sirens were kept individually in 30-gallon plastic tanks connected with a flow-through water system that kept the tanks ¾ filled with dechlorinated water at 22C. Each siren was fed bloodworms (4-6% of body weight at study onset) once every 3 days. Leftover worms and waste were removed from the tank every 3 days. During the study, animals were monitored twice daily (approximately every 12 hours +/- 2 hours) for signs of clinical disease including skin lesions (skin sloughing, ulcerations, proliferations) and behavioral changes (aberrant swimming, excessive hiding or failure to hide, hyporexia, etc).

Implant Placement

A total of eight sirens received implants, with four (Sirens 3, 5, 7, and 9) receiving terbinafine impregnated implants (24.5mg implant, Melatek LLC, Prairie du Sac, WI 53578 USA) and four (Sirens 2, 4, 6, and 8) receiving the base silicone elastomer implant with no terbinafine. A ninth animal (Siren 1) received no implant. Sirens were anesthetized with an immersion bath of benzocaine (200-400 mg/L). Appropriate anesthetic plane was determined by the loss of righting reflex and lack of response to stimuli. Because sirens are fully aquatic, they were placed in dorsal recumbency on a moistened towel and water was intermittently pipetted over their gills. A #15 scalpel blade was used to make a small stab incision in the ventrocaudal coelom, 3 centimeters cranial to the vent and just lateral to midline. The needle of the implant device was directed caudally into the coelomic cavity through the stab incision, and the implant was injected. After confirming the implant had been injected, the body wall was closed with 4-0 PDS in a single cruciate suture. The skin was dried, and cyanoacrylate tissue glue applied for apposition. Each animal received an intramuscular injection of Meloxicam (0.4-1.0mg/kg) for pain control. The animal was then transferred to its regular housing tank and monitored for recovery and the total time anesthetized was recorded.

Terbinafine assay

Animals were anesthetized as above monthly for blood collection to monitor plasma terbinafine levels. Blood was collected from the ventral tail vein using a 25 g needle on a 1 mL syringe, at a maximum volume equal to 1% of body weight. After collection, blood was

immediately transferred to lithium heparin tubes and placed on ice for processing. Within 3 hours, tubes were centrifuged and plasma stored at –80°C. Frozen samples were stored until all samples for the study had been collected (37-165 days) and then transported to the University of Tennessee Pharmacology Laboratory, where plasma terbinafine concentrations were measured by high-pressure liquid chromatography (HPLC) (Cox et al., 2015).

Bd exposure and diagnostics

All sirens tested negative for *Bd* immediately prior to exposure. *Bd* exposures for the eight animals that had implants occurred on day 35 following implant placement, using the isolate that was cultured from the positive sirens at intake (Bd-GPL1). The ninth animal was not exposed to *Bd*. After recovery from anesthesia for blood collection, each animal was transferred to a 3L container of water and $5x10^6$ *Bd* zoospores were pipetted directly onto the dorsal skin. The sirens were held for 24 hours in these inoculation tubs, and then released back into their tanks. Since all sirens tested PCR negative for *Bd* in the month following initial exposure, a second inoculation following the same procedure was performed on day 60 post-implant placement. Skin swabs were performed every 6 days and *Bd* PCR run as previously described.

Necropsy

Animals were anesthetized with the previously described method for final blood collection and skin swab on day 128 post-implant placement. Euthanasia was performed for all *Bd*-exposed (n = 8) animals using concentrated benzocaine to provide a ten-fold overdose. Exsanguination via cardiac puncture was used secondarily. After euthanasia, all animals were examined externally for lesions, and then a complete necropsy was performed, including visual identification of the implant in the coelomic cavity. Animals were then fixed in 10% neutral buffered formalin for histopathology. Trimmed sections of head, mid-body, feet, and tail were placed in tissue cassettes and processed. Histology slides were stained with H&E and examined under 400x magnification for evidence of *Bd* organisms and associated lesions.

Results

Animal health

Animals recovered well from each anesthetic event. All animals maintained normal appetites throughout the study and had no changes in activity levels. There were no gross skin lesions visible at any time point. However, all animals did experience weight loss over the study period, with an average of 10.7% loss (range 7-19%).

Two animals (siren 5 and 6) had surgical site dehiscence occur at days 11 and 12 post-implant placement. These individuals were anesthetized, and incisions were closed again as previously described. Recovery was without incident and no additional complications of implant placement occurred. Incision sites on all animals healed well, with minimal scarring visible at examination on day 35 postimplant placement (Figure 1, all tables and figures are shown in an appendix at the end of the chapter).

Plasma terbinafine

Terbinafine analysis required a minimum of 0.1 mL of plasma, therefore values were not determined for all animals at every time point due to difficulties in blood collection. All four of the terbinafine-implanted sirens had detectable concentrations of terbinafine in the plasma, while the blank-implanted sirens and the siren without any implant remained at zero (Table 1 and Figure 2). Only one animal (Siren 9) reached plasma terbinafine levels above the previously published MIC for *Bd* zoospores (63 ng/mL) (Woodward et al., 2014). Siren 9 had plasma levels above this benchmark at two sampling points – 102ng/mL at day 68 postimplant placement and 78ng/mL at day 98.

Bd PCR

All animals tested negative for *Bd* via qPCR in the month following the first exposure. Following the second exposure, low positive loads were detected in both the blank and terbinafine-impregnated implant groups (Figure 2). One animal (Siren 2) in the blank implant group had no positive swabs. Peak loads occurred 18-30 days following the second exposure, with two animals in each treatment group showing significant peaks. Sirens 3 and 7 in the

terbinafine-treated group had peak *Bd* loads of 49.9 copies/µL and 44.5 copies/µL respectively. Sirens 6 and 8 in the blank group had peak *Bd* loads of 64.27 copies/µL and 28.95 copies/µL. By day 60, all animals with blank implants tested negative, whereas all animals with terbinafine implants were either negative or had loads below 10 copies/µL.

Necropsy and Histopathology

No gross lesions were detected on necropsy and all animals appeared to be in good health. Implants were found in the caudal coelomic cavity, with no associated fibrous material, adhesions, or inflammation (Figure 4). Histopathology revealed no chytrid-associated lesions. Only one animal had *Bd* organisms present on histology (Siren 7, terbinafine group), with solitary organisms found on sections of the toes and mouth, without any associated inflammation or changes to the epidermis (Figure 5).

Discussion

The intracoelomic route for drug-impregnated implants was more successful than the subcutaneous route in this species, as the implants remained in place for all animals. Other than the two incidents of surgical site dehiscence, there were no detectable adverse effects from the implants. The terbinafine implants did not appear to greatly impact the development or clearance of *Bd* infection in the sirens. While the animal with the highest plasma terbinafine levels (Siren 9) had the lowest peak *Bd* load among the terbinafine-treated animals, there were two animals in the blank implant group that never developed significant *Bd* loads (Sirens 2 and 4). *Bd* loads overall were very low, no animals developed gross lesions of chytridiomycosis, and all animals cleared their infections at approximately the same rate, regardless of implant type. Why these animals were resistant to *Bd* infection is unclear. There are no formal studies in the literature on *Bd* susceptibility in this species, and individual reports of *Bd* infections are sparse, so it is possible that this species has low susceptibility. These animals were wild-caught and tested positive for *Bd* upon arrival at the lab, prior to heat treatment, and so may have maintained some level of immunity from prior infection, especially considering that the strain used for exposures was cultured from these same animals at intake.

The single animal with *Bd* organisms present on histology (Siren 7) was in the terbinafine-treated group. This animal was one of the two in the terbinafine-treated group that had a detectable spike in *Bd* load at day 18 post-second *Bd* exposure (day 86 post-implant placement) and had the highest *Bd* load at necropsy of any of the animals (9.7 copies/µL). Terbinafine levels in the plasma at the time of the peak *Bd* load in this animal would have been between 39 and 54 ng/mL, below the suggested MIC for terbinafine against *Bd* zoospores. The combination of waning terbinafine levels and a lower individual immunity to *Bd* than other animals in the study may explain the presence of organisms on this animal. Still, despite the presence of organisms, there were no clinical signs of chytridiomycosis and no associated microscopic lesions.

Great variability in plasma terbinafine concentrations were seen in this study, consistent with previous reports of terbinafine administration via implants in other species (Hardman et al., 2021; Kane et al., 2017). The mechanism behind this variability is unknown. While the plasma concentrations of terbinafine only went above the MIC for *Bd* zoospores in one animal, it is unknown what the concentrations were in the skin, where infection would be found, and where terbinafine has been shown to accumulate in other amphibians (Roberts et al., 2019). Additionally, for 3 of the 4 sirens with terbinafine implants, there was not enough blood collected at the first post-implantation timepoint (35 days) to run the terbinafine analysis, and for one individual this was also the case at the second blood collection (68 days). Since the peak terbinafine level in the animal that did have a sample for all four timepoints (Siren 5) occurred at 35 days, it is possible that terbinafine levels were higher at this time in the other animals as well. Overall, terbinafine levels detected in this study were lower than those found in hellbenders with the same implants injected subcutaneously (Hardman et al., 2021). This may be related to the route of administration, or to species differences in drug absorption. As the implants are intended as prophylaxis, lower concentrations may be sufficient to impede the growth of zoospores and prevent progression to disease. Future studies are recommended to investigate terbinafine levels in the skin, as well as different doses of terbinafine in the implants, and how this affects systemic absorption.

Animals lost an average of 10.7% of their body weight during the study, including Siren 1 (10.6% decrease in weight), which had neither received an implant nor been exposed to *Bd*. Therefore, weight loss in most of the animals is likely attributable to stress or inadequate diet. In the wild, these animals would be eating a wide variety of invertebrates, and so the diet of bloodworms alone in the lab may not have met their nutritional needs. The single animal that did lose more weight than the others, Siren 6 (19.4% weight loss) in the blank implant group, was also the individual with the highest peak Bd load (64.3 copies/μL). This may be evidence that this animal did develop clinical chytridiomycosis but was able to clear the infection without specific treatment, as by the end of the study it had 3 consecutive negative swabs, and no organisms present on histopathology.

While the efficacy of using intracoelomic terbinafine implants to prevent *Bd* chytridiomycosis remains unclear, the implants themselves appear to be a safe method of drug delivery using intracoelomic injection. Future studies investigating the use of intracoelomic implants in other large amphibian species, with different drugs, and aimed at identifying effective doses, are warranted. The use of these implants may provide a practical method for long-term drug delivery in free-ranging wildlife and in headstart programs where naïve animals are released into the wild.

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Appendices

Appendix A: Tables

Table 1.1 Plasma terbinafine levels determined by HPLC in 9 adult greater sirens at 4 time points over the course of 128 days post-injection of intracoelomic implants. Siren 1 received no implant, Sirens 2, 4, 6, and 8 received blank implants, and Sirens 3, 5, 7, and 9 received terbinafine-impregnated implants. Dashes indicate that insufficient plasma was available for terbinafine analysis at that timepoint.

Plasma Terbinafine (ng/mL)

	Siren 1	Siren 2	Siren 4	Siren 6	Siren 8	Siren 3	Siren 5	Siren 7	Siren 9
Days Post-Implant	No Implant	Blank Implant	Blank Implant	Blank Implant	Blank Implant	Terbinafine Implant	Terbinafine Implant	Terbinafine Implant	Terbinafine Implant
35	0	0	-	-	-	-	59	-	-
68	0	0	-	-	0	-	31	54	102
98	0	0	0	0	0	17	17	39	78
128	0	-	0	0	0	28	13	22	46

Appendix B: Figures

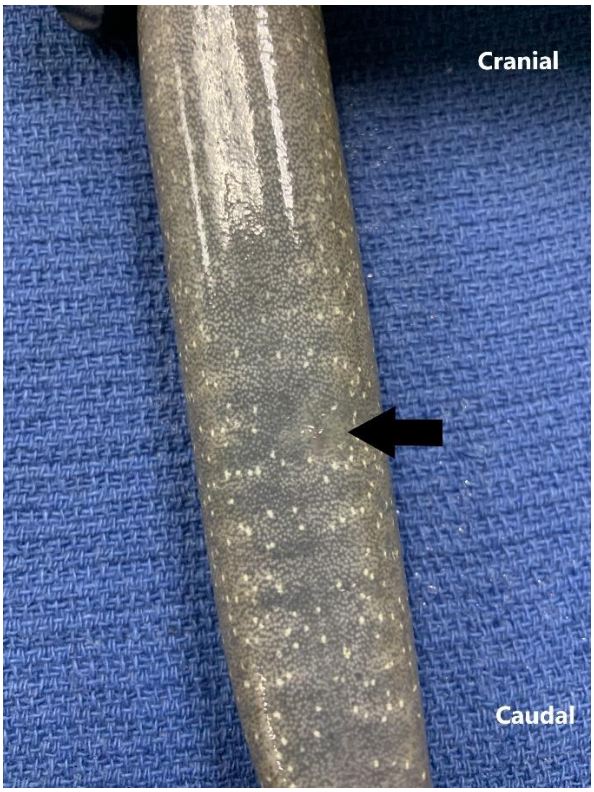

Figure 1.1 Image of healed implant site (black arrow) on the ventral caudal aspect of a greater siren, 35 days post-injection.

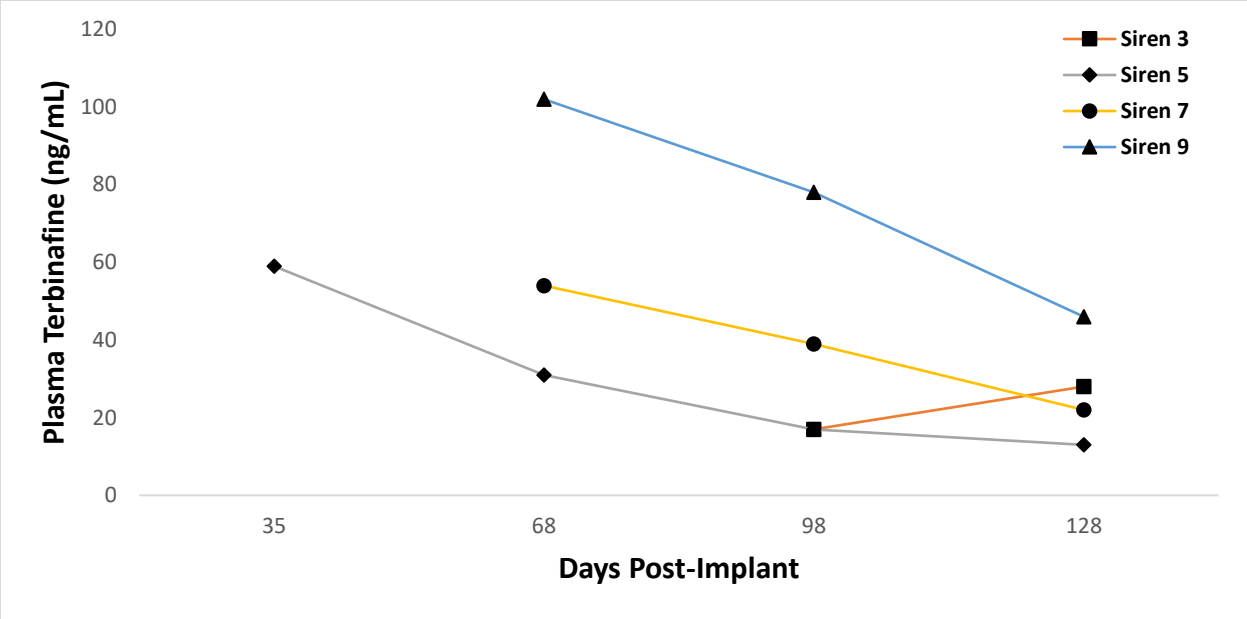

Figure 1.2 Line graph displaying plasma terbinafine concentrations determined by HPLC in four adult greater sirens over the course of 128 days postinjection of intracoelomic terbinafine-impregnated implants. Each line represents plasma concentrations for a single individual over time.

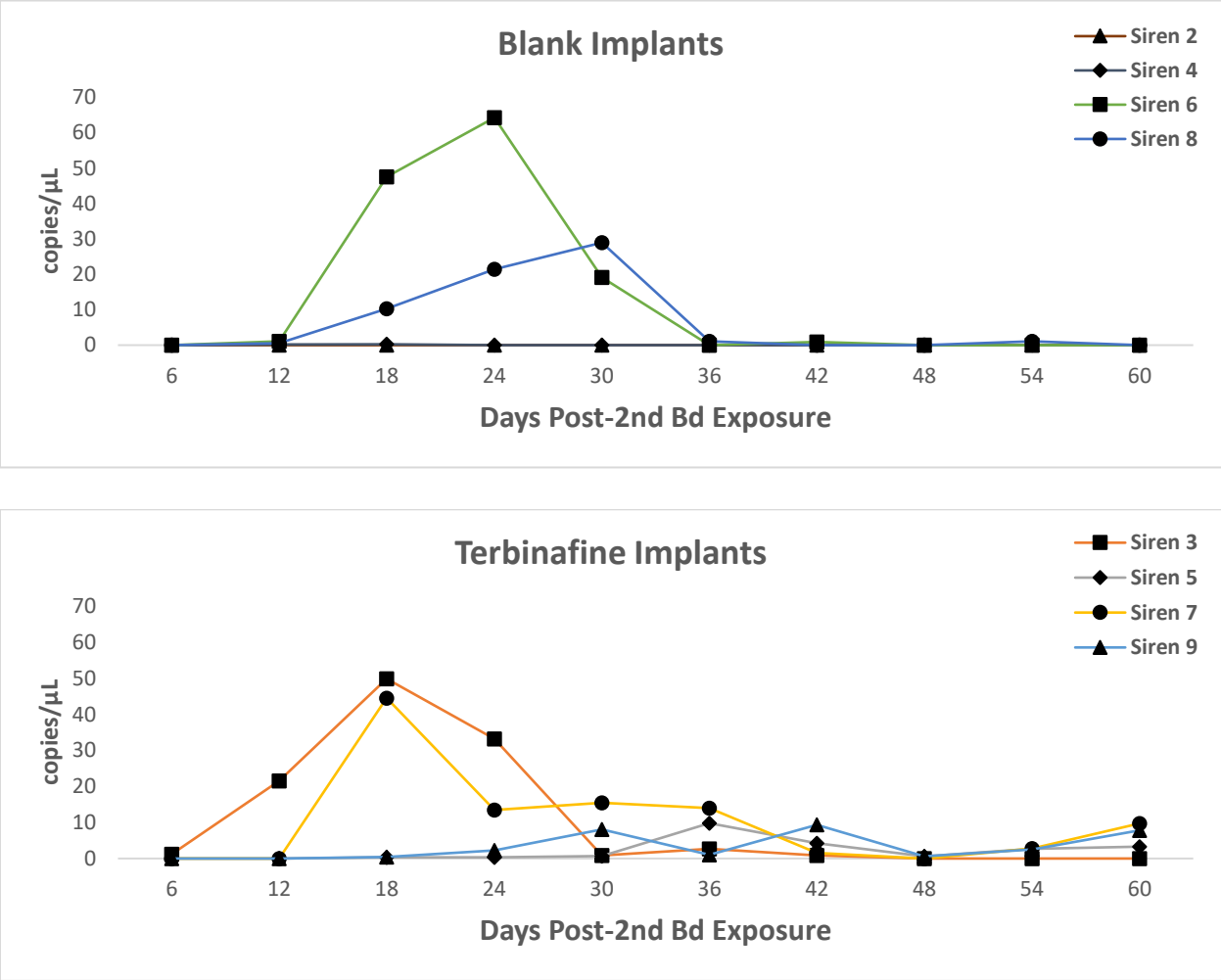

Figure 1.3 Individual *Batrachochytrium dendrobatidis* (*Bd*) load trajectories over time on adult greater siren skin collected from swabs and estimated with quantitative PCR after exposure to *Bd* zoospores. Two exposure events ($5x10^6$ zoospores at each event) occurred 30 days apart, with the graphs representing the swabs taken after the 2^{nd} exposure. The top graph represents individuals that received blank implants 30 days prior to the first *Bd* exposure, while the bottom graph represents individuals that received terbinafine implants.

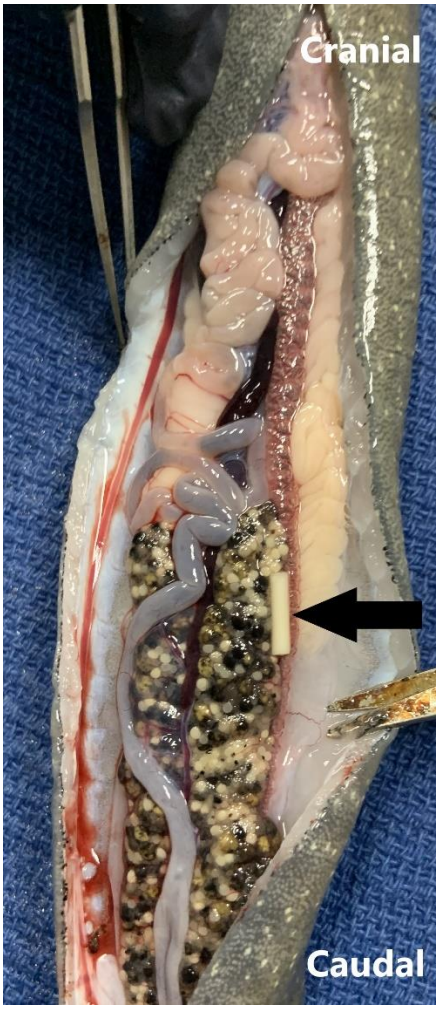

Figure 1.4 Image of terbinafine-impregnated implant (black arrow) in the coelomic cavity of a greater siren at necropsy, 128 days post-implant placement.

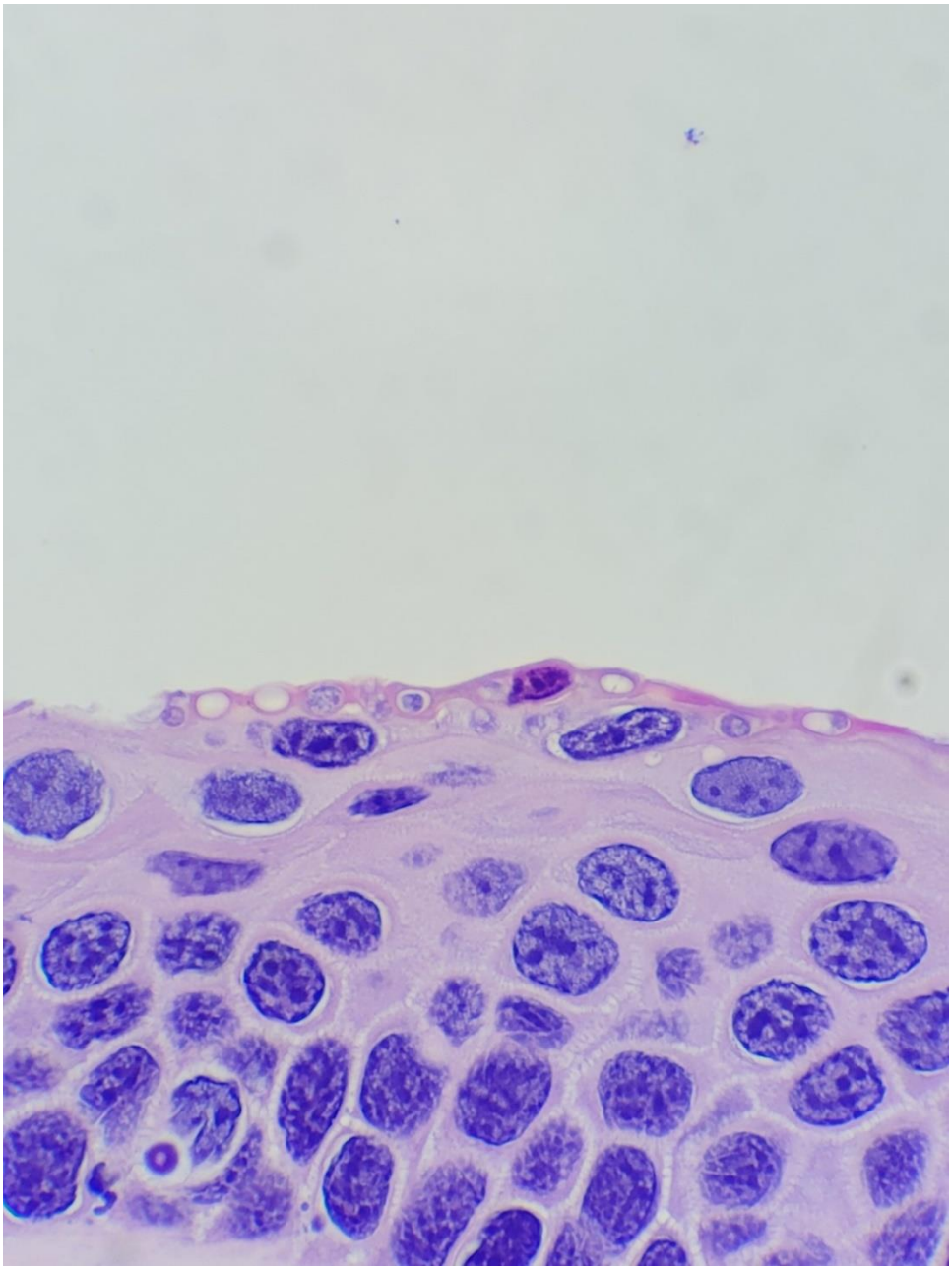

Figure 1.5 *Bd* organisms present in the superficial epidermis of a section of the toes of a greater siren (Siren 7), 128 days post-injection of a terbinafine-impregnated implant, and 60 days post-*Bd* exposure.

Chapter II: Risk of Bacteremia Associated with Probiotic Treatment of *Batrachochytrium salamandrivorans*

Abstract

Batrachochytrium salamandrivorans (*Bsal*) is a recently described fungal pathogen that has caused declines of wild fire salamanders (*Salamandra salamandra*) and been detected in captive populations in Europe. One potential preventative and treatment option for *Bsal* infection and the subsequent disease chytridiomycosis is the use of probiotic bacteria. While previous studies have shown that certain bacteria present on amphibian skin have anti-fungal properties, whether these bacteria have a significant positive or negative effect on host health and *Bsal* infection remains largely unknown. In this study, we had two objectives. First, we aimed to determine the safety and efficacy of the usage of probiotic bacteria (*Pseudomonas fluorescens*) isolated from the skin of newts to prevent and treat chytridiomycosis. Second, we tested whether bacteremia plays a role in the pathogenesis of chytridiomycosis. Eastern newts (*Notophthalmus viridescens*) were exposed to *Bsal*, probiotic bacteria, pathogenic bacteria (*Aeromonas hydrophila*), or a combination of *Bsal* and one of the bacterial species. For the latter, animals were either exposed to bacteria a week prior to or at one of two timepoints after *Bsal* exposure. Newts were monitored throughout the course of disease development and samples were collected for *Bsal* and bacterial PCR, blood cultures, and histopathology. Our results indicated that *Bsal* loads on the skin measured by PCR were lower in some groups exposed to probiotic bacteria, but the difference was not statistically significant among probiotic treatment groups. Among the low dose *Bsal* groups, only the group exposed to *A. hydrophila* prior to *Bsal* exhibited a difference in survival, with a mortality rate of 67%. Histopathologic lesion counts and lesion grades did not differ among groups that were exposed to *Bsal*. We also found evidence of bacterial infiltration into the blood and organs in animals that were exposed to *Bsal* in probiotic-exposed and -unexposed newts, with increased *P. fluorescens* loads in the blood and organs of probiotic-exposed newts. Neither blood cultures nor PCR detected *A. hydrophila* in the blood or organs of the pathogen-exposed newts. Our results suggest that the probiotic bacteria, *P. fluorescens*, was not able to prevent or treat *Bsal* infection. Future studies should further examine the potential for probiotics to contribute to bacteremia and Bsal pathogenesis.

Introduction

Batrachochytrium salamandrivorans (*Bsal*) is a relatively recently discovered chytrid fungus that infects amphibians (Martel et al., 2013) and is related to the more well-known chytrid fungus *B. dendrobatidis* (*Bd*). The first detected cases of *Bsal* chytridiomycosis were in fire salamanders in the Netherlands, where populations experienced 96% mortality rate from 2010 to 2013 (Spitzen-Van Der Sluijs et al., 2013). *Bsal* may have been responsible for a die-off in Germany as early as 2004 (Lötters, Veith, et al., 2020).

The current hypothesis is that *Bsal* originally co-evolved with salamanders in East Asia, and was introduced to Europe through the pet trade (Stegen et al., 2017). Thereafter, it rapidly spread through Europe, now present in 80 sites in 4 countries (Lötters, Wagner, et al., 2020). It is not known to occur in North America (Waddle et al., 2020), but some North American species are susceptible, including the widely distributed and abundant eastern newt (*Notophthalmus viridescens*) (Martel et al., 2014). The US, especially the southeast, is a global biodiversity hotspot for salamander species. Vulnerability models based on habitat suitability, species richness, and major ports for salamander import have predicted serious consequences for North American salamander biodiversity if *Bsal* arrives (Yap et al., 2015)

Bsal causes lesions that involve degrading the integrity of the skin. This leads to erythema, skin sloughing, ulcers that often form characteristic circular "target" lesions, hemorrhage, and even the loss of toes and tail tip (Martel et al., 2013). These lesions interrupt the major functions of the skin: osmoregulation and respiration. As most salamander species rely to some extent on cutaneous respiration, these impacts can be deadly. *Bsal* infects the upper layers of the epidermis, causing necrotic lesions that can infiltrate through to the dermis. This creates a potential pathway for secondary infection. One current theory is that septicemia from these secondary infections is a major contributor to cause of death (Bletz et al., 2018).

Potential treatment options are a major area of *Bsal* investigation. Environmental fungicides have been used for other pathogens, such as *Bd.* These include chemical compounds like Virkon, as well as plant-derived compounds like curcumin, calendula, oregano, and cloves. Several of these compounds have shown promising inhibitory and fungicidal effects against *Bsal*

zoospores *in vitro* as well (Tompros, Wilber, et al., 2022), but the use of these compounds in the environment and for treating infected individuals is still being explored. Individual host treatment protocols have also been developed. For example, *Bsal* infected fire salamanders treated with topical polymyxin E and voriconazole at 20°C for 10 days successfully cleared *Bsal* infections (Blooi et al., 2015). While both environmental and individual fungicidal treatments show promise, there are also downsides to these options when considering treatment of wild populations. The impact of fungicides on the health of the ecosystem must be considered, and there may be unintended negative impacts on other organisms in the environment. Meanwhile, individual repeated dosing of fungicides for infected hosts may not be practical for free-ranging animals.

A more recent area of study is the use of probiotics, using organisms that already contribute to the salamanders' innate immune defenses to prevent and treat diseases like chytridiomycosis. Salamanders naturally have a low abundance of microbiota on their skin, including some *Bsal*-inhibitory members (Woodhams et al., 2007). *Bsal* infection disrupts this microbial community, leading to an increase in opportunistic bacteria; this may potentiate septicemic events, as bacteria have previously been cultured from the livers of *Bsal* infected animals, and not from uninfected controls (Bletz et al., 2018; Latorre et al., 2019). Probiotic treatment may have the benefit of restoring the natural balance of the microbial community, as well as augmenting members of this community that produce antifungal compounds. This method has been successful in the treatment of *Bd* in the lab. Augmenting the naturally occurring *Janthinobacterium lividum* bacterium on the skin of mountain yellow-legged frogs (*Rana mucosa*) with a *J. lividum* isolate known to produce anti-*Bd* metabolites prevented the lethal effects of disease after *Bd* exposure (Harris et al., 2009). Though all frogs in this study had the bacterium on their skin, the metabolite produced by *J. lividum*, violacein, was found only on the frogs bathed in the *J. lividum* solution, whereas frogs not treated had no detectable violacein on their skin. This result suggests that bioaugmentation with suitable anti-*Bd* bacteria can be used to prevent *Bd* infection.. However, it should be noted that *in situ*, a single probiotic treatment did not have a protective effect against *Bd* (Knapp et al., 2022). While probiotics have shown variable promise in treating *Bd*, their ability mitigate *Bsal* infection remains largely unexplored.

In this study, *Pseudomonas fluorescens was* selected as our candidate probiotic. *P. fluorescens* is a common gram-negative bacterium consisting of a relaxed species group of organisms that are found in soil, water, and in the natural skin microbiota of amphibians (Lam et al., 2010; Nikolaidis et al., 2020). Isolates of *P. fluorescens* cultured from *R. muscosa* skin showed a synergistic effect with antimicrobial peptides produced by the frog in their activity against *Bd* in vitro (Myers et al., 2012) and *Pseudomonas* isolated from eastern red-backed salamanders (*Plethodon cinereus*) have also shown antifungal activity (Lauer et al., 2007). An isolate of *P. fluorescens* from newt skin was therefore chosen as a potential probiotic in this study. For our pathogenic bacteria, we used the gram-negative bacteria *Aeromonas hydrophila*, which is found in healthy amphibians (Hird et al., 1981) and can cause opportunistic infections (Hubbard, 1981). It is the most common species of *Aeromonas* that causes infections in animals such as amphibians, reptiles, and fish, usually causing hemorrhagic septicemia (Huang et al., 2015). *A. hydrophila* served as a representative of the members of the newt microbiota that might opportunistically contribute to disease in the immunocompromised state caused by *Bsal* chytridiomycosis.

We had two objectives in this study. The first objective was to determine the safety and efficacy of using probiotic bacteria to prevent and treat *Bsal* chytridiomycosis on *N. viridescens*. The second objective was to evaluate bacteremia and septicemia as components of morbidity and mortality caused by *Bsal* chytridiomycosis in *N. viridescens*. Ultimately, our goal was to use clinical signs, mortality rates, *Bsal* infection intensities, histopathology, and blood and organ cultures to comprehensively test the capacity of bacteria to affect (improve or reduce) the health of animals suffering from *Bsal* infection.

Methods

Animal Husbandry

One hundred forty *Notophthalmus viridescens* (eastern newt, NOVI) adults were captured in the wild in Tennessee, USA and transported to the Johnson Animal Research and Teaching Unit at University of Tennessee, Knoxville. They were housed individually in 1L containers filled with approximately 300mL of dechlorinated water and a PVC cover object. We fed animals

bloodworms (2% of their body mass every 3 days) and replaced each animal's container, cover object, and water every 3 d. Animals were kept in environmental chambers on a 12-h light, 12-h dark cycle, at >90% humidity. Temperature was gradually raised by 2°C per day from ambient temperature to 30°C and then held for 10 d to clear any pre-existing Bd infections (Chatfield & Richards-Zawacki, 2011). The temperature was then lowered by 2°C per day to 14°C, the approximate optimal growth temperature *Bsal* (Martel et al., 2013), and the animals were acclimated to this temperature for 2 weeks.

Bacterial and *Bsal* Growth

An isolate of *Pseudomonas fluorescens* cultured from the skin of wild NOVI was provided by the Woodhams lab at University of Massachusetts (Boston, MA). A Kwik-Stik™ pack of *Aeromonas hydrophila* derived from ATCC® 7966™ was purchased from Microbiologics. Growth curves were performed for *A. hydrophila* and *P. fluorescens* at 14C to correspond to the temperature that the animals used in the study would be kept at during bacterial exposure. Optical density measurements were recorded hourly until 0.5 OD600 was reached, and then continued every 2-4 hours. Serial dilution and plating were performed once 1 OD600 was reached and time to this growth point was recorded. Plates were incubated at 14°C and colony counts performed once visible colonies arose. Inoculation cultures were grown for the appropriate time to reach 1 OD600 (22 hours for *P. fluorescens* and 8 hours for *A. hydrophila*), confirmation optical density measurements were performed, and the cultures were diluted to 10^8 CFU/mL. Serial dilution and plating were also performed on the inoculation cultures for colony count confirmation.

Bsal (isolate AMFP13/1) used in this study was obtained from the Netherlands (Martel et al., 2013). The cultures were maintained at the University of Tennessee Center for Wildlife Health laboratory. *Bsal* zoospores used for exposure were harvested from tryptone gelatin hydrolysate (TGhL) agar plates after 6 d of growth. Plates were flooded with 7 mL of autoclaved dechlorinated water and filtered with a 20-µm sieve to isolate zoospores. Zoospores were enumerated using a hemocytometer and verified using flow cytometry (Carter et al., 2020).

Bacteria and *Bsal* Exposure

Animals were randomly assigned to twelve treatment group. Treatment groups were as follows: (1) Negative Controls (n=13); (2) *Bsal* Controls (n=11); (3) *Pseudomonas* controls (n=9); (4) *Aeromonas* Controls (n=9); (5) *Pseudomonas* Pre- *Bsal* Exposure (n=9); (6) *Aeromonas* Pre-*Bsal* Exposure (n=9); (7) *Pseudomonas* Post-*Bsal* Exposure (n=16); (8) *Pseudomonas* Post- High Dose *Bsal* Exposure (n=9); (9) *Aeromonas* Post-*Bsal* Exposure (n=16); (10) *Aeromonas* Post- High Dose *Bsal* Exposure (n=9); (11) *Pseudomonas* Post-*Bsal* Lesions (n=16); (12) *Aeromonas* Post-*Bsal* Lesions (n=14). Two animals originally included in group 12 died prior to *Aeromonas* exposure, and were therefore recategorized as group 2.

We exposed animals per their treatment group in 100-mL plastic cylindrical containers with 9 mL of autoclaved dechlorinated water and 1 mL of the randomly assigned *Bsal* zoospore dose. All *Bsal* exposures occurred on the same day. Groups 2, 5-7, 9, 11, and 12 were all exposed to the "low" dose of *Bsal* ($5x10^3$ zoospores) while groups 8 and 10 were exposed to the "high" dose of *Bsal* ($5x10^5$ zoospores). The high dose groups were included to determine whether there was an effect of *Bsal* dose on the efficacy of probiotic treatment or development of secondary infections. Control animals were treated identically but exposed to 10 mL of autoclaved dechlorinated water. The bacterial control groups (3 and 4) were exposed only to their respective bacteria on the same day that *Bsal* exposures occurred for the other treatment groups and were transferred to autoclaved dechlorinated water after 3 hours of bacterial exposure. After 24 h, we removed newts from the inoculation tubes and placed them back into their tubs.

All bacterial exposures were approximately 10^8 CFU for 3 hours in 100-mL plastic cylindrical containers with 9mL of autoclaved dechlorinated water and 1 mL of the assigned bacterial broth. As noted, the bacterial control groups (3 and 4) were exposed the same day that *Bsal* exposures occurred for the other treatment groups. The pre-exposure groups (5 and 6) were exposed to their respective bacteria 6 days prior to *Bsal* exposure. The post-*Bsal* exposure groups (7-10) were exposed to their respective bacteria 6 days after *Bsal* exposure. The post-*Bsal* lesions groups (11 and 12) were exposed to their respective bacteria after animals in those groups started to develop gross signs of chytridiomycosis, at day 20 post-*Bsal* exposure. After the bacterial exposure, we removed newts from the inoculation tubes and housed them individually as

previously described. The timeline of study events is presented in Figure 1 (all tables and figures are shown in an appendix at the end of each chapter).

Monitoring, Necropsy, and Diagnostic Procedures

We monitored twice daily for signs of *Bsal* chytridiomycosis, including lethargy, focal lesions, ulcerations, increased skin sloughing, hemorrhage, anorexia, and loss of righting response (Carter et al., 2020; Martel et al., 2013). Animals were humanely euthanized when they lost righting ability, and recorded as a mortality event for data analyses. We swabbed each animal every 6 d starting 4 d post-exposure to *Bsal*, using standardized swabbing protocols for *Bd* and *Bsal* (Blooi et al., 2013).

A subset of groups 7 and 9 (n=7 from each group) were euthanized 6 days after bacterial exposure to allow for examination for transient bacteremia that may not have been detectable by the time the animals reached *Bsal* disease endpoints. The same procedure was planned for group 11 and 12, however 7 animals from each group had already died or been euthanized prior to the scheduled euthanasia date. At necropsy, animals were designated for culture (n=93) or histopathology (n=47). Animals in the culture diagnostic group were necropsied and blood was collected from the heart for culture, and the liver, spleen, and heart were collected for bacterial PCR. A necropsy swab of the skin as well as a skin and toe sample were also collected for *Bsal* PCR. The body was then preserved in an individual whirl-Pak bag in 10% neutral buffered formalin. Animals in the histopathology diagnostic group were necropsied and a swab, skin sample, and toe sample were collected for *Bsal* PCR. The animals were preserved in individual whirl-Pakbags in formalin and transferred to 70% ethanol after 24 hours.

Quantitative PCR (qPCR) for *Bsal* was run on each necropsy swab and other selected swabs following methods described in Carter et al (2020). Quantitative PCR for *Pseudomonas* and *Aeromonas* were also run on liver samples from animals in the culture diagnostic group, as initial runs of *Pseudomonas* PCR were most consistent on liver samples. Blood cultures were performed within 12 hours on all blood samples collected from the animals in the culture diagnostic group. Histological cross-sections of epidermal tissues stained with hematoxylin and eosin were examined for *Bsal* lesions in the animals from the histopathology diagnostic group.

Lesions were counted and graded using the following scale for depth and extent: (1) stratum corneum; (2) mid-epidermis; (3) full thickness epidermis; (4) full thickness, coalescing/large lesion less than or equal to 1mm in length, and (5) full thickness coalescing/large lesion greater than 1mm in length. Perimeter of each section was measured using Excelis Accu-Scope software to standardize measures of lesion counts to total perimeter of all sections for each animal.

Statistical Analyses

All statistical analyses were performed in RStudio version 2022.12.0. Survival data was analyzed by performing Kaplan-Meier survival analysis and Cox proportional hazard models with the *survival* package (v3.5-5; Therneau 2023). We performed an analysis of variance (ANOVA) comparing log-transformed *Bsal* zoospore copies per microliter postmortem among treatment groups. Assumptions of normality and homoscedasticity were evaluated with diagnostic plots. When the analysis of variance was significant ($\alpha = 0.05$), post hoc Tukey Honestly Significant Difference (Tukey HSD) tests were performed to evaluate pairwise differences among treatment groups. For bacterial load analysis by PCR and culture, we fitted a binomial regression to predict the presence or absence of bacteria based on treatment group. We then performed an ANOVA conditional on presence to compare bacterial loads between treatment groups. Lesion counts and grades were modeled as count data with treatment group as the fixed effect and section perimeter as the offset term, and *p*-values were computed using a Wald z-distribution approximation.

Results

Survival Analysis

Mortalities occurred in all *Bsal*-exposed groups independent of bacterial treatment (*Pseudomonas, Aeromonas)*, and no mortality was observed in the *Bsal*-unexposed groups. There were low numbers of survivors (range 11-33%) in all groups exposed to low-dose *Bsal* and bacteria, but survival did not significantly differ from the low-dose *Bsal* control group in any of the *Pseudomonas* treated groups (Chisq= 1.8 on 3 degrees of freedom, p= 0.6, Figure 2.2). However, the group that was treated with *Aeromonas* 6 days prior to *Bsal* exposure had a 67% mortality rate (Figure 2.3), which was significantly less than the 100% mortality rate in the *Bsal*

control group (Chisq= 8.1 on 3 degrees of freedom, p= 0.04). Median survival time among all groups exposed to low-dose *Bsal* was in the 22-25 day range with the exception of the group exposed to *Aeromonas* prior to *Bsal* exposure, where median survival time was 31 days. Rapid mortalities occurred in both groups exposed to high-dose *Bsal*, with all animals reaching euthanasia endpoints or dying by 10 days post-*Bsal* exposure.

Comparison of *Bsal* Loads by PCR

Bsal loads by qPCR performed on necropsy swabs in the *Bsal* control group were used as a comparison point for all groups exposed to *Bsal* and bacteria (Figure 2.3 and 2.4). Within each group, there was wide variation in *Bsal* loads at necropsy. Among the *Pseudomonas* groups, the highest *Bsal* loads were in the group exposed to *P. fluorescens* prior to *Bsal* exposure (mean = 12.99, sd = 1.3 log(Copies/uL +1)) and in the group that received the higher dose of *Bsal* (mean = 12.02, sd = 2.75 log(Copies/uL +1)). The lowest loads were in the groups that were exposed to *Pseudomonas* six days after low-dose *Bsal* exposure (mean = 8.68, sd = 3.4 log(Copies/uL +1)) and after the development of *Bsal* lesions (mean = 8.59, sd = 5.31 log(Copies/uL +1)). The *Bsal* loads among the *Aeromonas* groups were overall similar to each other. Despite apparent trends in the *Pseudomonas* groups, the ANOVA revealed no significant differences in *Bsal* loads between groups in either the *Pseudomonas* (F= 2.13, df = 4, p =0.090) or *Aeromonas* (F = 0.94, df = 4, p = 0.449) study.

Pseudomonas Loads by qPCR and Blood Culture

Estimates of *Pseudomonas* loads in liver tissue and *P. fluorescens* in blood from the *Pseudomonas*-exposed groups were provided by qPCR and culture, respectively, and compared to loads in the three control groups (Figure 2.6 and 2.7). Based on qPCR, there was one positive animal in the negative control group, and otherwise, positive results were only found in groups with both *Bsal* and *P. fluorescens* exposure. The binomial GLM for presence of *Pseudomonas* on qPCR by treatment group showed substantial explanatory power (Tjur's R2 = 0.31), however there was not a significant difference in presence of *Pseudomonas* between any of the treatment groups and the negative control group. The model's intercept, corresponding to the negative control group, is at -1.61. Within this model: the effect of low dose *Bsal* only is statistically non-

significant and negative (beta = -16.96, p = 0.995), the effect of *Pseudomonas* treatment only is statistically non-significant and negative (beta =-16.96, p = 0.995), the effect of *Pseudomonas* treatment prior to *Bsal* is statistically non-significant and positive (beta = 1.61, p = 0.278), the effect of *Pseudomonas* treatment after *Bsal* is statistically non-significant and positive (beta = 0.76, p = 0.556;), the effect of *Pseudomonas* treatment after high-dose *Bsal* is statistically non-significant and positive (beta = 3.00, p = 0.056), and the effect of *Pseudomonas* treatment after the development of *Bsal* lesions is statistically non-significant and positive (beta = 2.01, p = 0.113). The ANOVA for PCR load in *Pseudomonas*-positive animals suggested that the effect of treatment group was not statistically significant (F = 0.67, df = 4, $p = 0.624$).

Blood culture results for *P. fluorescens* were highly variable within treatment groups (Figure 2.7). One animal in the *Bsal* control group had a positive blood culture. Otherwise, positive cultures were only found in groups with both *Bsal* and *P. fluorescens* exposure. The binomial GLM for the presence of a positive culture based on treatment group had substantial explanatory power (Tjur's R2 = 0.44). The model's intercept, corresponding to the *Bsal* control group, is -1.39. Within this model: the effect of *Pseudomonas* treatment after the development of *Bsal* lesions is statistically significant and positive (beta = 2.77, $p = 0.043$), the effect of *Pseudomonas* treatment after high-dose *Bsal* is statistically non-significant and positive (beta = 2.77, $p = 0.080$), the effect of *Pseudomonas* treatment after *Bsal* is statistically non-significant and positive (beta = 1.79, $p = 0.165$), the effect of *Pseudomonas* treatment prior to *Bsal* is statistically non-significant and positive (beta = 2.48, p = 0.122), the effect of *Pseudomonas* treatment only is statistically non-significant and negative (beta =-17.18, $p = 0.995$), and the effect of no exposure or treatment (negative control) is statistically non-significant and negative (beta = -17.18, $p = 0.995$). The highest loads were in the group that was exposed to *Pseudomonas fluorescens* after the development of *Bsal* lesions (mean = 4.54 $x10^4$, sd = 3.696 $x10^4$ CFU/mL), though this was not significantly different from the other groups that had animals with positive blood cultures (F = 0.792, df = 4, $p = 0.546$).

There was no evidence of transient bacteremia in the subset of group 7 animals that were euthanized 6 days after bacterial exposure. PCR and blood culture values were lower on average

in this subset (2.83 copies/uL and $2.0x10^3$ CFU/mL) than in the animals in the same treatment group that died or were euthanized because of disease (6.96 copies/uL and $3.1x10^4$ CFU/mL).

Aeromonas hydrophila load analysis was not performed for the *Aeromonas*-exposed groups because both culture and PCR failed to detect the organism.

Histopathology

Lesion Counts

All treatment groups exposed to *Bsal* had lesions present on histologic examination, while those not exposed to *Bsal* had no lesions. Lesion counts among groups exposed to Bsal are presented in Figure 2.8 and 2.9. The *Bsal* control group had the highest overall lesion counts per mm (mean = 0.96, sd = 0.62). Among the *Pseudomonas*-exposed groups, those exposed to *Pseudomonas* after low-dose *Bsal* had the lowest lesion count per mm (mean = 0.48, sd = 0.5) while the group exposed to *Pseudomonas* prior to *Bsal* had the highest (mean = 0.94, sd = 0.39). The Poisson model of lesion counts by treatment group was overdispersed, therefore a negative binomial model was used, with the treatment group as the predictor variable and the section perimeter in micrometers as the offset term. The model's intercept, corresponding to the *Bsal* control group at a section perimeter of 0µm, is at 2.78. Within this model: the effect of *Pseudomonas* treatment prior to *Bsal* is statistically non-significant and negative (beta = -0.12, p = 0.821), the effect of *Pseudomonas* treatment after *Bsal* is statistically non-significant and negative (beta = -0.97, p = 0.059), the effect of *Pseudomonas* treatment after high-dose *Bsal* is statistically non-significant and negative (beta = -0.14, p = 0.780), and the effect of *Pseudomonas* treatment after *Bsal* lesions is statistically non-significant and negative (beta = -0.32, p = 0.483).

Two animals in group 9 (exposed to *Aeromonas* 6 days following low-dose *Bsal* exposure) had minimal lesions – one had zero and one had a single lesion. Among the *Aeromonas* exposed groups, this was the group with the lowest lesion counts per mm (mean = 0.416, sd = 0.4). The group that was exposed to *Aeromonas* prior to *Bsal* exposure had the highest lesion count per mm of the *Aeromonas* groups (mean = 0.81, sd = 0.145). Due to overdispersion in the Poisson model, a negative binomial model was fit to the lesion counts with

treatment group as the predictor variable and section perimeter in µm as the offset term. The model's intercept, corresponding to *Bsal* control and section perimeter= 0, is at 2.79. Within this model: the effect of *Aeromonas* prior to *Bsal* exposure is statistically non-significant and negative (beta = -0.44, p = 0.580), the effect of *Aeromonas* after *Bsal* exposure is statistically non-significant and negative (beta = -1.12, p = 0.115), the effect of *Aeromonas* after high-dose *Bsal* is statistically non-significant and negative (beta = -1.12, p = 0.162), and the effect of *Aeromonas* following the appearance of *Bsal* lesions is statistically non-significant and negative (beta = -0.82, p = 0.306).

Lesion Grade

Lesion grades were evaluated as density (count per total cross section perimeter for each animal) of each grade (Figures 2.9 and 2.10) . Grade 1 lesions were most common in the *Bsal* control group. Grade 2 lesions were most common in the *Bsal* control group, the group exposed to *Pseudomonas* prior to *Bsal* exposure, and the group exposed to *Pseudomonas* after *Bsal* lesions developed. Grade 3 lesions were most common in the groups that were exposed to bacteria prior to *Bsal* exposure, regardless of which bacteria they were exposed to. Grade 4 and 5 lesions were the least common of all lesion grades and were found in approximately equivalent density across treatment groups. When pairwise comparisons were made between treatment groups that were exposed to the same species of bacteria, and between each *Bsal*-exposed group and the *Bsal* control group, treatment group did not have a significant effect on lesion grade.

Organ Analysis

Select slides from individuals exposed to bacteria were Gram stained for examination of blood vessels and internal organs for infiltration of Gram-negative rods, however no organisms were consistently visible. One animal in group 11 (*Pseudomonas* exposure following development of *Bsal* lesions) had parasitic cysts in the liver, corresponding with multifocal white nodules observed on gross necropsy.

Discussion

The main objectives of this study were to determine the safety and efficacy of using probiotic bacteria to prevent or treat chytridiomycosis, and to evaluate bacteremia and septicemia as components of morbidity and mortality caused by chytridiomycosis. Probiotic treatment with *Pseudomonas fluorescens* did not confer significant protection from morbidity and mortality caused by *Bsal* chytridiomycosis. While there were individuals within the probiotic-treated groups that survived for the duration of the study, and a few of these individuals cleared their infections, this has been seen in previous studies with NOVI exposed to 10^3 zoospores of *Bsal* at 14C (Carter et al., 2021). Interestingly, the only low-dose *Bsal*-exposed group with significantly different survival was the group exposed to our pathogenic bacteria, *A. hydrophila*, prior to *Bsal* exposure. This same effect was not seen in groups that were exposed to *A. hydrophila* after *Bsal* exposure. Prior studies have shown that *A. hydrophila* produces several bioactive compounds, some of which have antifungal activity (Kamal et al., 2017). It is possible that *A. hydrophila* had some inhibitory effect on *Bsal* pathogenesis in these individuals, though overall *Bsal* loads and lesion counts were not different from the other groups. The median survival time of newts exposed to high-dose *Bsal* was shorter (10 days) than has been seen previously (16 days in Carter et al., 2021). Bacterial exposure shortly after *Bsal* exposure may have contributed to more rapid mortalities in this case, as conditions were otherwise the same as in the prior study. Neither *Bsal* loads at necropsy, nor total lesion density or density by grade showed any difference among the various *Bsal*-treated groups.

The most significant finding in evaluation for bacteremia and septicemia among the probiotic-treated groups was the increased prevalence of *P. fluorescens* found by blood culture in the animals that were treated after developing *Bsal* lesions. This indicates that the lesions did provide a pathway for bacteria to infiltrate into the blood and organs. Despite a lack of statistical significance between groups, positive PCR and blood culture were rare in the three control groups (1 animal positive for each), compared to the groups that were exposed to both the *P. fluorescens* and *Bsal*. This trend could indicate that the combination of exposures led to bacteremia and septicemia in those individuals. *Pseudomonas* loads by PCR and culture did not always seem to agree on the same animal. This is likely because the PCR assay was for the

broader *Pseudomonas* genus, while blood cultures were able to detect the *P. fluorescens* species group. There were some cases where blood cultures were positive and PCR was negative, which could be due to bacteria not yet colonizing the liver, or the section of liver that was tested, at detectable levels. There is a possibility that *Pseudomonas* infection may have originated from salamander microbial flora. Determining whether the *P. fluorescens* found in the blood was the same strain as the one that was used for probiotic treatment was not within the scope of this study and was not pursued.

There were two significant limitations in this study that prevent stronger conclusions about the contribution of bacteremia and septicemia to *Bsal* pathogenesis. One is the failure of the diagnostic tests for *A. hydrophila*. It is unclear if the organism was truly absent from the blood and organs of the animals that were exposed to it, or if the liver PCR and blood cultures were just unable to detect it. Also, while the initial *A. hydrophila* exposure dose was known for these animals, later testing for bacterial loads on the skin was not performed. It is therefore difficult to fully evaluate*A. hydrophila* and *Bsal* exposure interaction in these groups. Despite the significant increase in survival in animals exposed to *A. hydrophila* prior to *Bsal* exposure, the extent to which this can be attributed to the bacterial treatment remains unclear. The other major limitation is that increased bacterial infiltration in the blood and liver, as seen in the group 11 animals, could not be correlated with a difference in survival. More specific histopathologic staining would be useful to aid in visualizing *P. fluorescens* and *A. hydrophila* in the blood vessels and tissues, and determining whether there are any associated microscopic signs of disease.

Overall, the findings from this study suggest that *Pseudomonas fluorescens* as administered in this study is not an effective probiotic against *Bsal* chytridiomycosis, regardless of the timing of treatment. While *Bsal* loads tended to be lower in animals exposed to *P. fluorescens* after low-dose *Bsal* exposure, this was neither statistically significant, nor did it seem to affect mortality rate or *Bsal* lesions. Based on the liver PCR and blood culture results, exposure to *P. fluorescens* either before or after *Bsal* exposure may lead to increased infiltration of bacteria in the blood and organs, with the greatest increase occurring when skin lesions are already present. There may indeed be a push and pull mechanism of *P. fluorescens* causing a

mild decrease in *Bsal* load, but also contributing to immunocompromise and illness in the animal, and therefore contributing to overall mortality. There still remain many candidate members of the salamander microbiome that could be used for probiotic treatment, and future studies should explore the use of different organisms or even a combination of organisms. Currently, probiotics seem most useful in captive animals where the microbiome can be controlled and have proven more difficult to use in free-ranging settings. As these protocols continue to be explored and refined, the findings in this study indicate that some caution should be exercised when exposing immunocompromised animals, especially those with skin lesions, to bacteria that may opportunistically exhibit pathogenic activity.

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Appendix

Appendix A: Figures

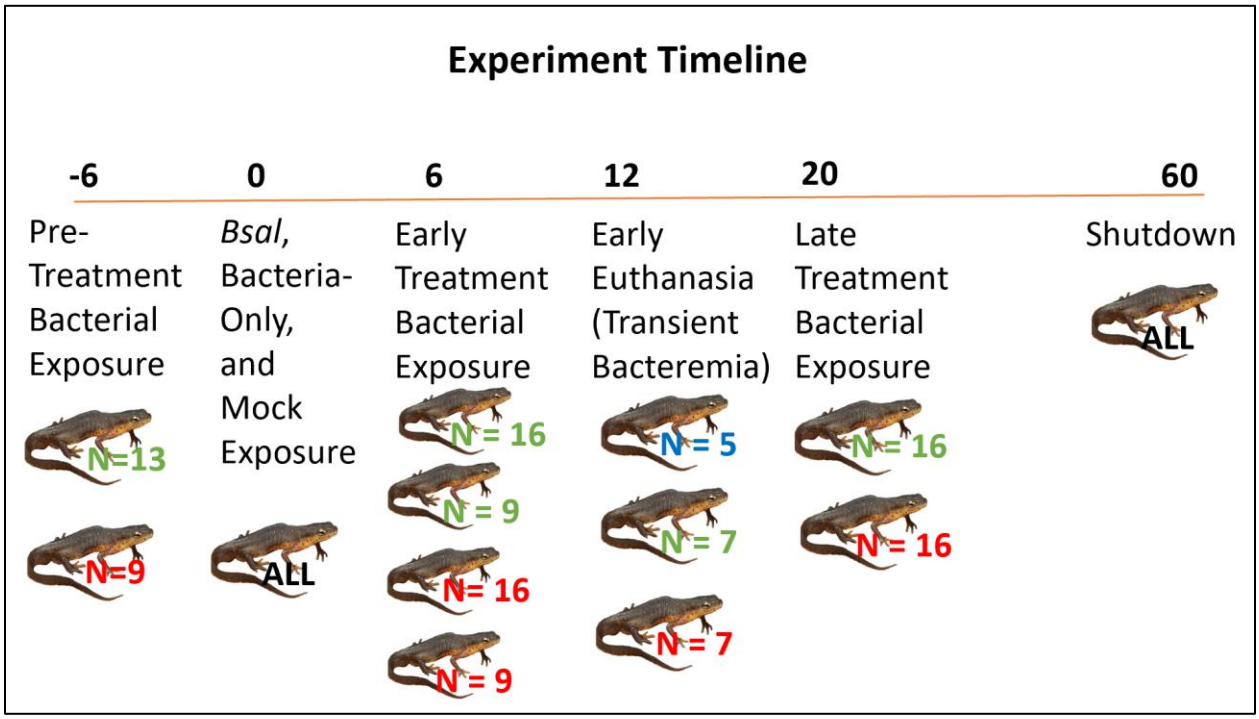

Figure 2.1 Timeline of study events, with the day of *Bsal* exposure designated at Day 0. Numbers in blue represent negative controls, green represent groups exposed to *Pseudomonas fluorescens*, and red represent groups exposed to *Aeromonas hydrophila*.

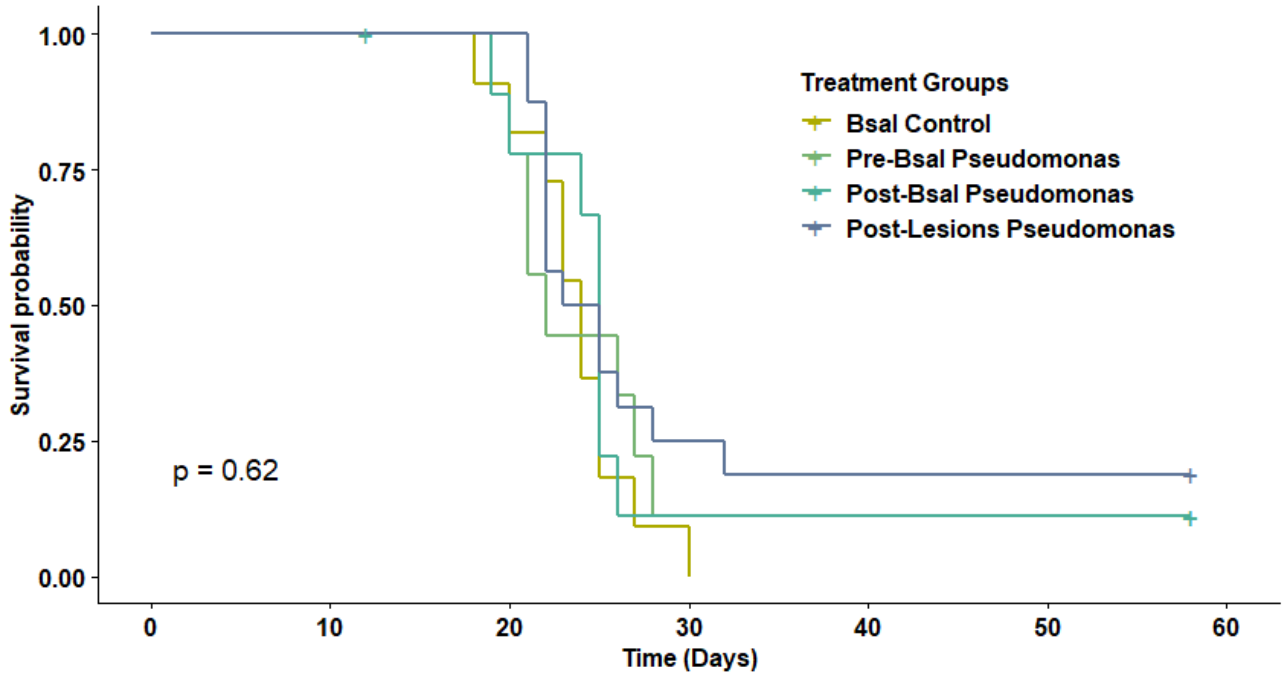

Figure 2.2 Kaplan-Meier survival curves representing the mortality rate of *Notophthalmus viridescens* exposed to one of four combinations of *Bsal* and *P. fluorescens* (*Bsal* only, *P. fluorescens* prior to *Bsal*, *P. fluorescens* after *Bsal*, and *P. fluorescens* after the development of *Bsal* lesions). The *p* value of the log-rank Kaplan-Meier test was 0.62, indicating no significant difference in survival between treatment groups.

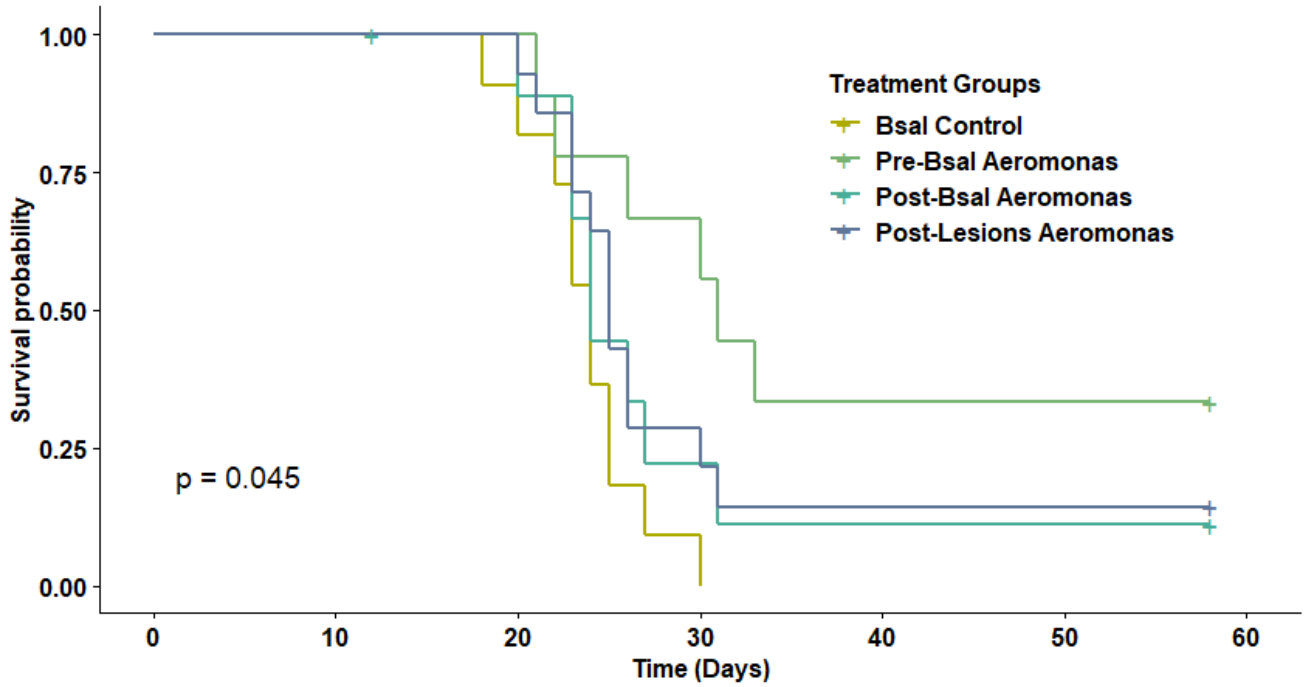

Figure 2.3 Kaplan-Meier survival curves representing the mortality rate of *Notophthalmus viridescens* exposed to one of four combinations of *Bsal* and *A. hydrophila* (*Bsal* only, *A. hydrophila* prior to *Bsal*, *A. hydrophila* after *Bsal*, and *A. hydrophila* after the development of *Bsal* lesions). The *p* value of the log-rank Kaplan-Meier test was 0.045, indicating a significant difference in survival between groups, with the group exposed to *A. hydrophila* prior to *Bsal* exposure exhibiting the highest survival probability.

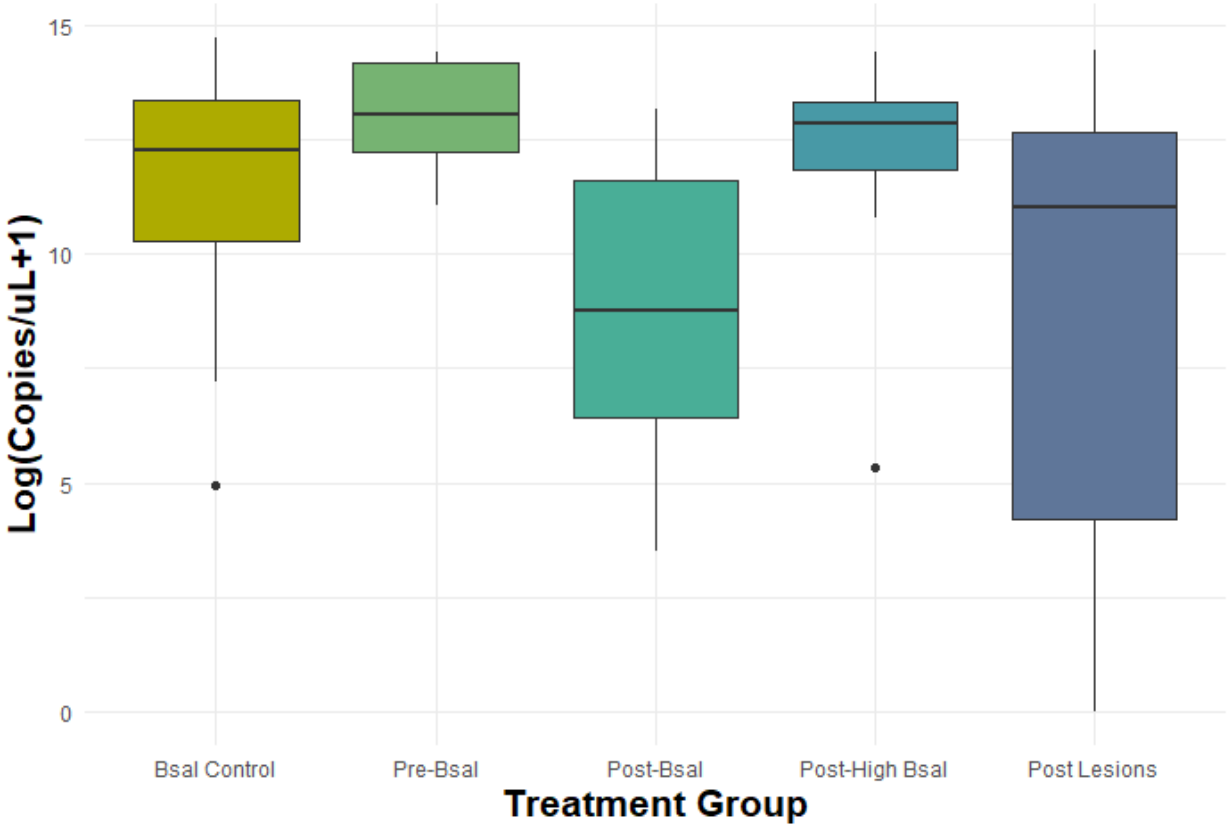

Figure 2.4 Mean *Bsal* load (copies/uL) at postmortem examination on eastern newt (*Notophthalmus viridescens*) skin collected from swabs and estimated with quantitative PCR. Treatment groups represent one of five combinations of *Bsal* and *Pseudomonas fluorescens* exposure (*Bsal* only, *P. fluorescens* prior to *Bsal*, *P. fluorescens* after *Bsal*, *P. fluorescens* after high dose *Bsal*, and *P. fluorescens* after the development of *Bsal* lesions).

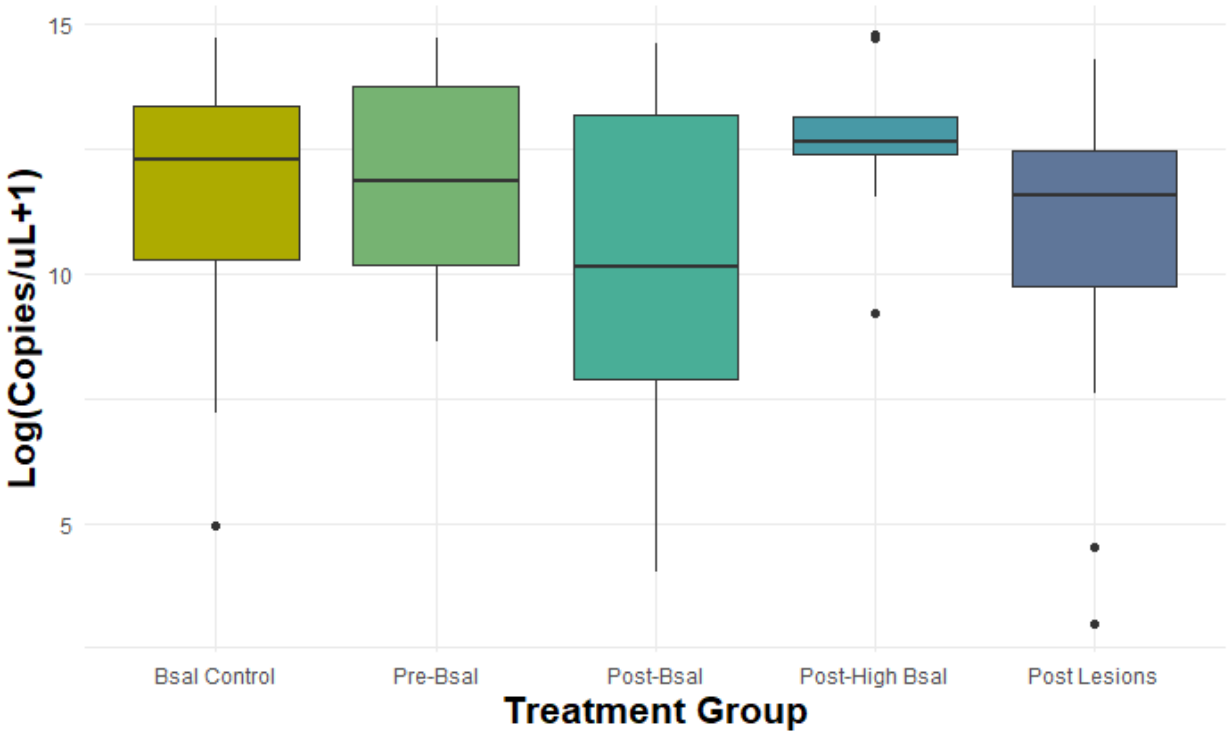

Figure 2.5 Mean *Bsal* load (copies/uL) at postmortem examination on eastern newt (*Notophthalmus viridescens*) skin collected from swabs and estimated with quantitative PCR. Treatment groups represent one of five combinations of *Bsal* and *Aeromonas hydrophila* exposure (*Bsal* only, *A. hydrophila* prior to *Bsal*, *A. hydrophila* after *Bsal*, *A. hydrophila* after high dose *Bsal* and *A. hydrophila* after the development of *Bsal* lesions).

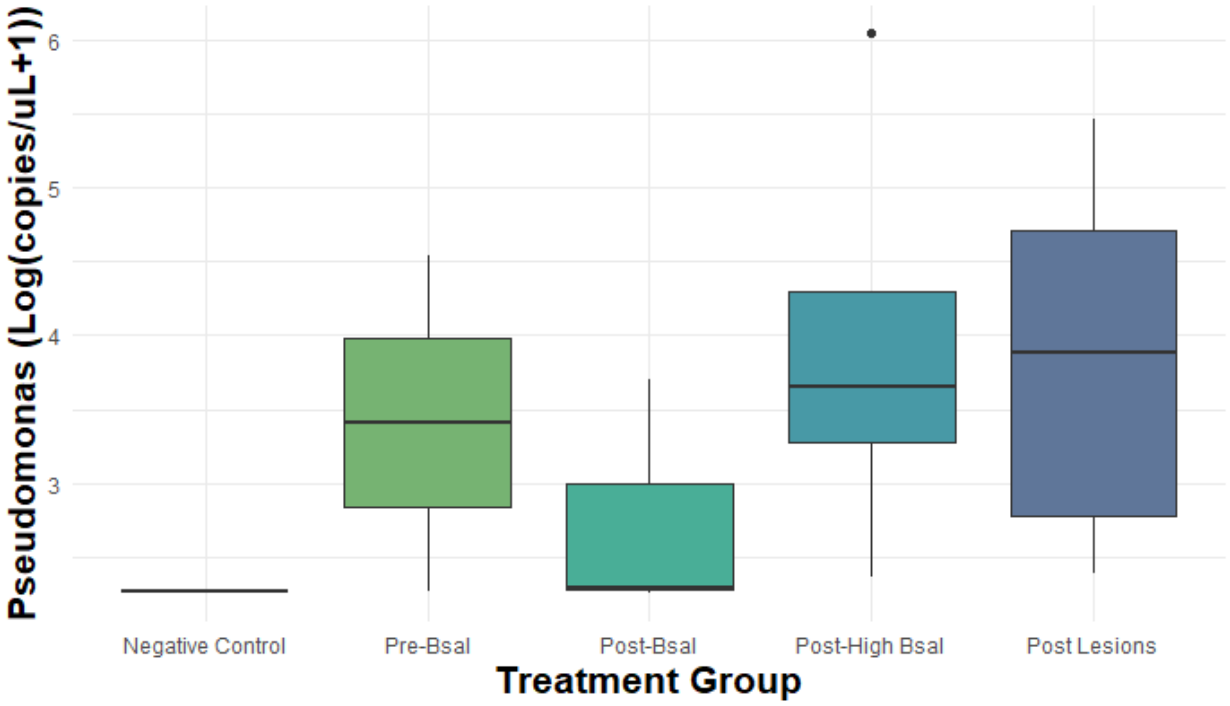

Figure 2.6 Mean *Pseudomonas* load (copies/uL) at postmortem examination on eastern newt (*Notophthalmus viridescens*) liver samples estimated with quantitative PCR. Only treatment groups with PCR-positive animals are represented. Treatment groups represent one of five combinations of *Bsal* and *Pseudomonas fluorescens* exposure (No exposure, *P. fluorescens* prior to *Bsal*, *P. fluorescens* after *Bsal*, *P. fluorescens* after high dose *Bsal*, and *P. fluorescens* after the development of *Bsal* lesions). There were no PCR-positive animals in the *Bsal* control or *Pseudomonas* control groups.

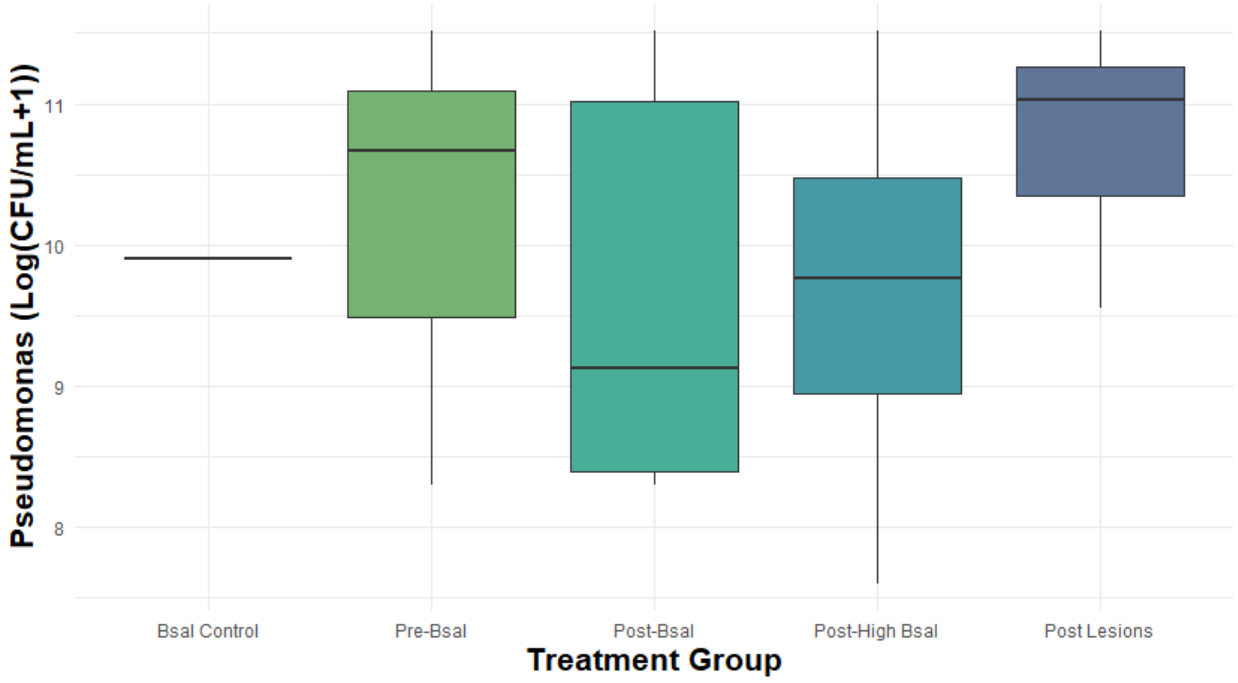

Figure 2.7 Mean *Pseudomonas fluorescens* colony counts (CFU/mL) at postmortem examination on eastern newt (*Notophthalmus viridescens*) blood cultures. Only treatment groups with culture-positive animals are represented. Treatment groups represent one of five combinations of *Bsal* and *Pseudomonas fluorescens* exposure (*Bsal* only, *P. fluorescens* prior to *Bsal*, *P. fluorescens* after *Bsal*, *P. fluorescens* after high dose *Bsal*, and *P. fluorescens* after the development of *Bsal* lesions). There were no culture-positive animals in the negative control or *Pseudomonas* control groups.

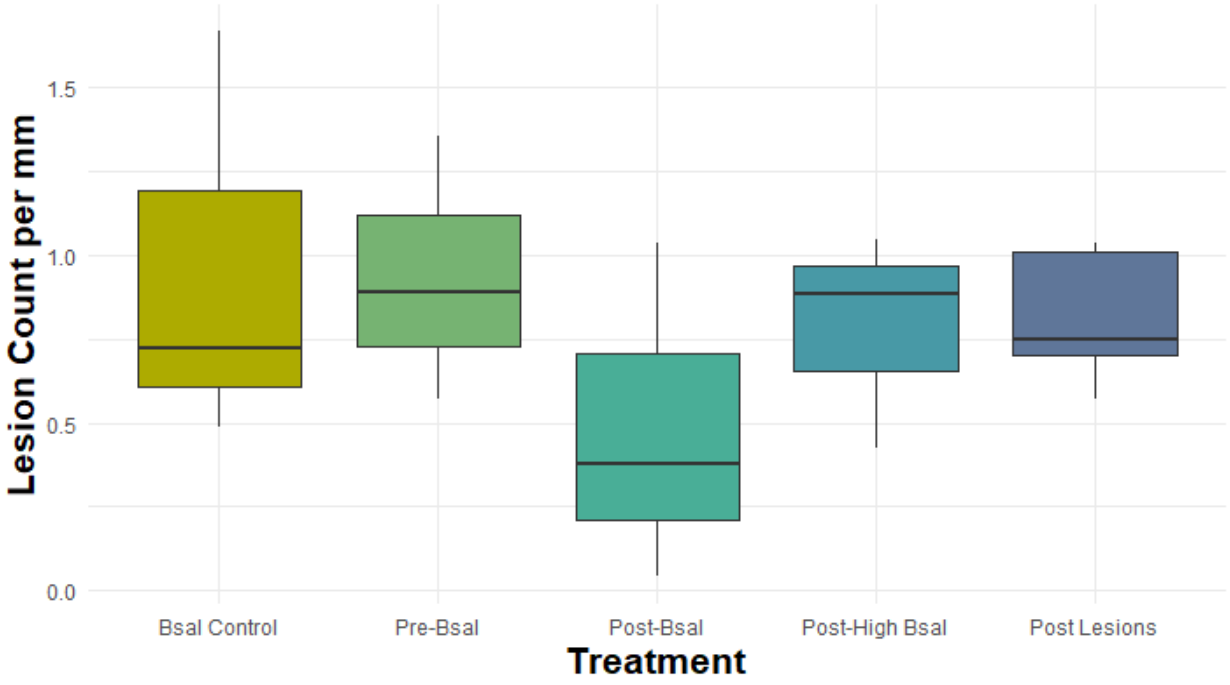

Figure 2.8 Mean *Bsal* lesion counts per mm at histopathologic examination on eastern newts (*Notophthalmus viridescens*). Treatment groups represent one of five combinations of *Bsal* and *Pseudomonas fluorescens* exposure (*Bsal* only, *P. fluorescens* prior to *Bsal*, *P. fluorescens* after *Bsal*, *P. fluorescens* after high dose *Bsal*, and *P. fluorescens* after the development of *Bsal* lesions).

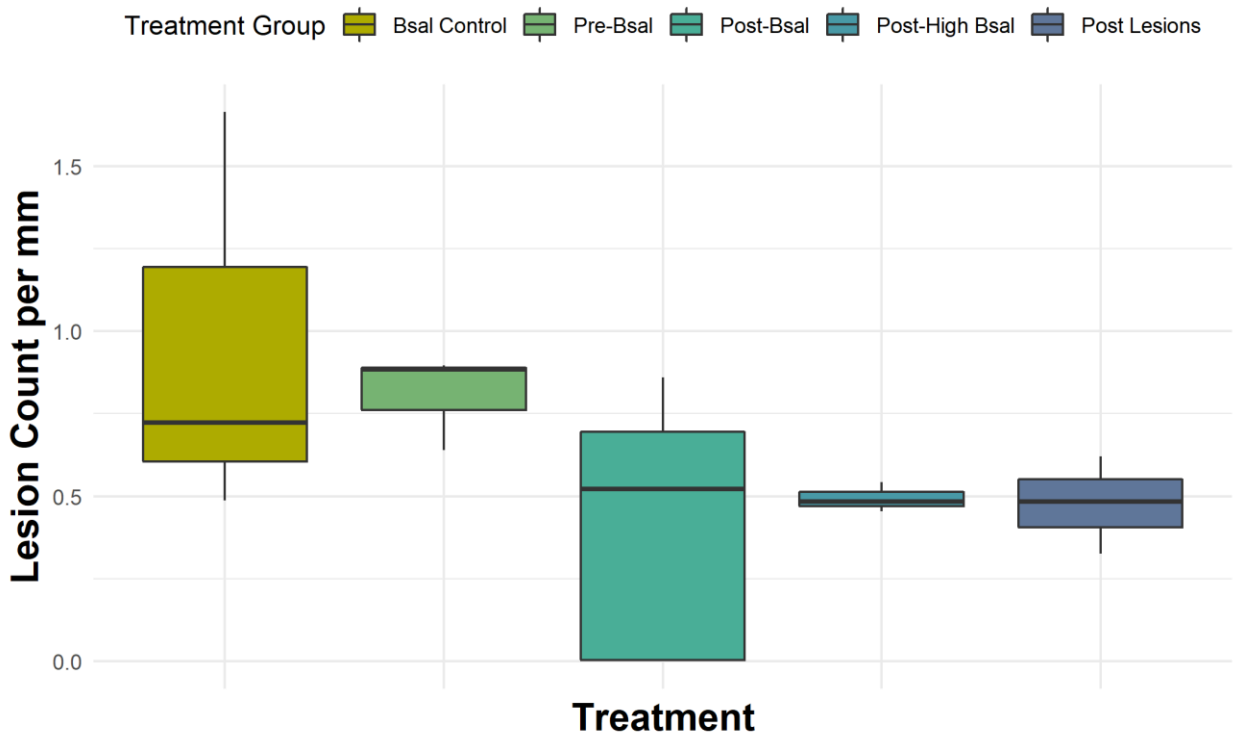

Figure 2.9 Mean *Bsal* lesion counts per mm at histopathologic examination on eastern newts (*Notophthalmus viridescens*). Treatment groups represent one of five combinations of *Bsal* and *Aeromonas hydrophila* exposure (*Bsal* only, *A. hydrophila* prior to *Bsal*, *A. hydrophila* after *Bsal*, *A. hydrophila* after high dose *Bsal* and *A. hydrophila* after the development of *Bsal* lesions).

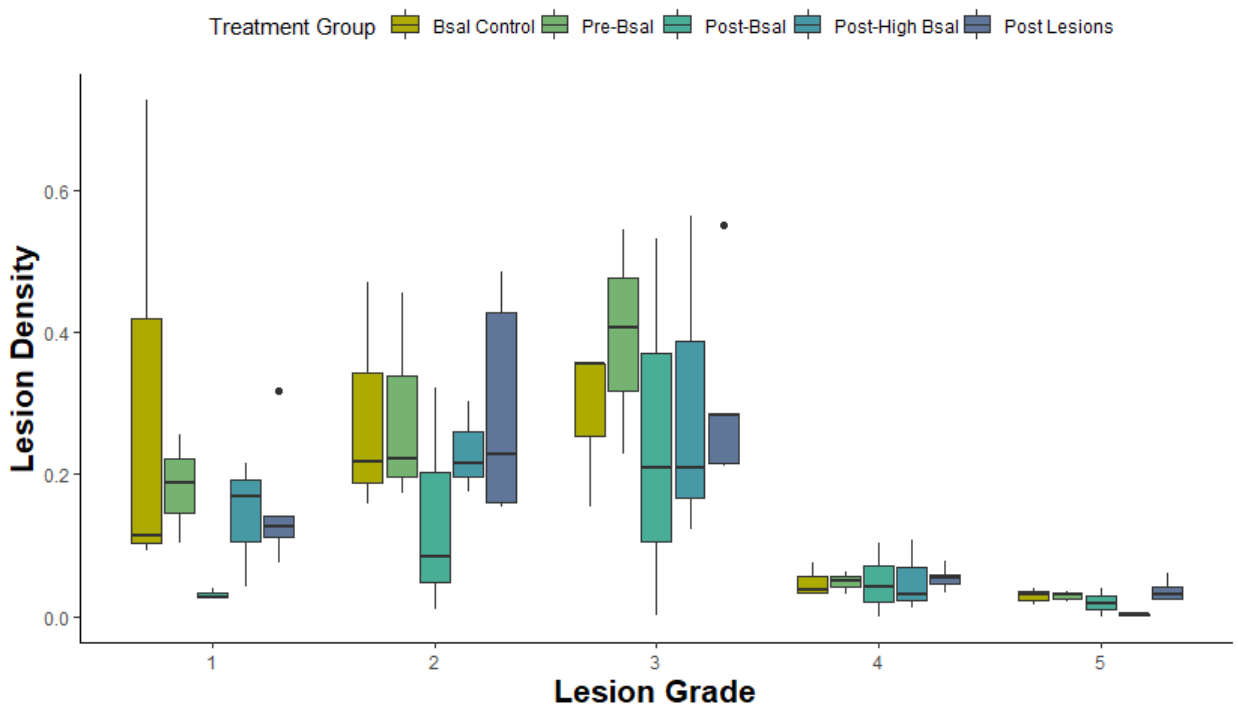

Figure 2.10 Plot of the average lesion density per unit of cross section (y-axis) for each lesion grade (x-axis) at histopathologic examination on eastern newts (*Notophthalmus viridescens*). Treatment groups represent one of five combinations of *Bsal* and *Pseudomonas fluorescens* exposure (*Bsal* only, *P. fluorescens* prior to *Bsal*, *P. fluorescens* after *Bsal*, *P. fluorescens* after high dose *Bsal*, and *P. fluorescens* after the development of *Bsal* lesions).

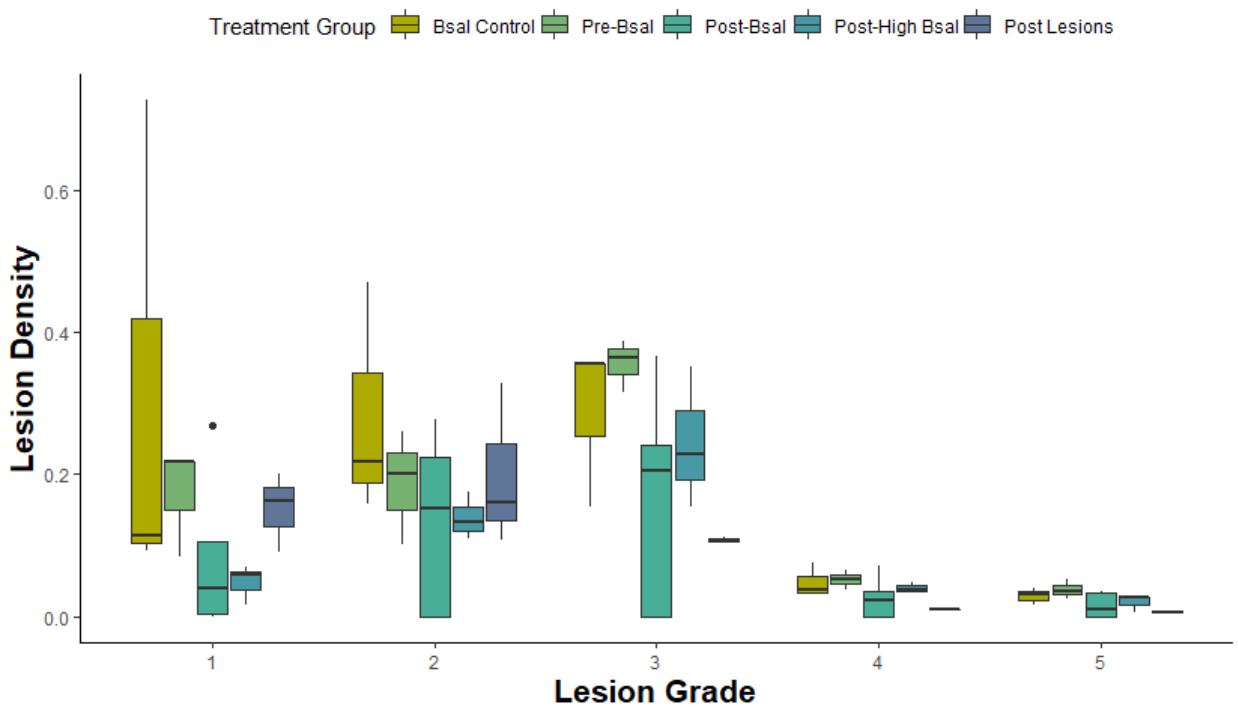

Figure 2.11 Plot of the average lesion density per unit of cross section (y-axis) for each lesion grade (x-axis) at histopathologic examination on eastern newts (*Notophthalmus viridescens*). Treatment groups represent one of five combinations of *Bsal* and *Aeromonas hydrophila* exposure (*Bsal* only, *A. hydrophila* prior to *Bsal*, *A. hydrophila* after *Bsal*, *A. hydrophila* after high dose *Bsal*, and *A. hydrophila* after the development of *Bsal* lesions).

Chapter III: Impact of *Bactrachochytrium salamandrivorans* Exposure on Eastern Newt Reproductive Fitness

Abstract

Emerging fungal pathogens have become an increasing threat to wildlife over the past several years. *Batrachochytrium salamandrivorans* (*Bsal*) is one such pathogen of amphibians and has led to species declines in several areas in Europe. Though it is not yet known to be present in North America, it does pose a substantial threat to vulnerable salamander populations and overall amphibian diversity. In the case of the eastern newt (*Notophthalmus viridescens*), the high risk of morbidity and mortality in the acute infection period is known, but there is little information yet on what sublethal effects *Bsal* exposure would have on a population. Understanding the effects of exposure on individuals that avoid mortality is important for targeted surveillance and mitigation efforts in North America.

The goal of this project was to expose eastern newts to *Bsal* and compare their reproductive fitness to unexposed controls through objective measures of reproductive quality and output, obtained through necropsy and histopathology. Individuals were either unexposed, exposed once, or exposed twice and were also euthanized at two different time points – during the "acute" time period, 30 days after their latest exposure, or during the "chronic" time period, 60 days after that exposure. A subset of individuals placed in male-female pairs was observed for breeding behaviors. We documented a decrease in ovary mass relative to body mass and an increase in testicular mass relative to body mass over time, though this trend did not significantly differ between exposure groups. The proportion of eggs with yolk development was greater in females that were exposed to *Bsal*, and in those that lived longer, with the greatest effect seen in the double-exposure group at 90 days post-exposure. Germinal epithelial depth in the males was also greatest in the double-exposure group. These parameters are potential indicators of terminal investment in this species with multiple *Bsal* exposures. This could have important implications for population dynamics in the face of *Bsal* invasion.

Introduction

While many wildlife groups are currently in decline, amphibians are among the most threatened, with nearly 60% of species in danger of extinction over the last 40 years (García-Rodríguez et al., 2022). There are multiple contributors to amphibian population declines, including habitat loss, environmental toxins, and infectious disease. Chytridiomycosis is a cutaneous fungal disease caused by two related fungi, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*). Whether caused by *Bsal* or *Bd*, chytridiomycosis disrupts the integrity of the skin, leading to systemic disease through the detrimental effects on hydration, ion exchange, and cutaneous respiration. Excessive water loss leads to dehydration and electrolyte imbalances, and, in *Bd* chytridiomycosis, this has been shown to lead to cardiac arrest and death (Salla et al., 2018; Voyles et al., 2009a; Wu et al., 2019). The same may be true for *Bsal* cytridiomycosis as a recent study reported similar detrimental effects on electrolyte balance in salamanders having deep ulceration and necrosis of the skin caused by *Bsal* (Sheley et al., 2023). It has also been shown that this skin erosion and ulceration promotes secondary bacterial overgrowth that may result in lethal septicemia (Bletz et al., 2018).

Chytridiomycosis has led to an amphibian panzootic that represents the greatest recorded disease-related loss of biodiversity, affecting over 500 amphibian species (Scheele, Pasmans, Skerratt, Berger, Martel, Beukema, Acevedo, Burrowes, Carvalho, Catenazzi, De La Riva, et al., 2019). While *Bd* is present in many areas around the world, *Bsal* is not yet known to occur in North America, with its current distribution restricted to Europe and Asia (Waddle et al., 2020). In Western Europe, *Bsal* has contributed to the near extirpation of *Salamandra salamandra* populations (Martel et al., 2013). *Bd*'s emergence as a global pathogen has been linked to the expansion of the commercial trade in amphibians (O'Hanlon et al., 2018) and there is a high likelihood that *Bsal* could be spread the same way. *Bsal* has been detected in the pet trade, and there are concerns that this could be the introduction point for the pathogen to currently naïve populations (Nguyen et al., 2017). With continued global movement of amphibians, *Bsal* poses a significant threat to North American amphibian biodiversity, and many species have been shown to be susceptible through laboratory trials (Carter et al., 2020; Friday et al., 2020; North American Bsal Task Force, 2020).

Research over the last decade has focused on a wide variety of aspects of *Bsal* pathogenesis and epidemiology, in a joint effort to address the devastation it has already caused in Europe and prevent these same results when it arrives in North America (Carter et al., 2020; Lötters, Wagner, et al., 2020; Martel et al., 2013; Stegen et al., 2017; Thomas et al., 2018). Part of these efforts includes modeling the potential distribution of *Bsal* in new areas and conducting studies that predict regions of particular susceptibility (Katz & Zellmer, 2018; Yap et al., 2017). However, adult morbidity and mortality are only part of the story when it comes to predicting population declines; sublethal effects that include recruitment of the next generation must be considered as well. In the case of *Bd*, previous studies have investigated these effects, including gross and histologic changes to gonads in infected animals (Brannelly et al., 2021), long-term impacts on body fat and gonad size in susceptible species (Campbell et al., 2019), and impacts on mating behaviors even after the infection has been cleared (An & Waldman, 2016). Field studies on *Bd* have also been incorporated into population level modeling to explain the extinction of some and persistence of other populations in the face of chytridiomycosis outbreaks (Briggs et al., 2005).

Whether related to disease status or not, stress is known to negatively impact reproductive hormone levels in amphibians (Kindermann et al., 2017; Moore & Zoeller, 1985). However, a direct relationship between these hormonal changes and changes in courtship, mating, and reproductive success has not been elucidated. While it stands to reason that the energy costs of disease and a decrease in reproductive hormones would lead to a decrease in reproductive fitness, studies in *Bd* have often revealed the opposite. These have demonstrated an increase in mating call effort in the Japanese tree frog (*Hyla japonica*) (An & Waldman, 2016) and an increase in testes width in the northern leopard frog (*Lithobates pipiens*) (Chatfield et al., 2013). These studies support what is known as the terminal investment hypothesis, when resources are reallocated from infection clearance and survival to reproductive output, thought to occur mainly in iteroparous animals (Clutton-Brock, 1984). Meanwhile, in the common mistfrog *(Litoria rheocola*), there was an increase in mating call effort during *Bd* infection when paired with good body condition, whereas poor body condition in the face of infection led to a decrease

in mating call effort (Roznik et al., 2015). Rather than supporting terminal investment across the board, this study suggested a more complex selection pressure, where individuals that were infected but still clinically healthy showed more reproductive effort even than uninfected individuals, while the more severely affected individuals perhaps had less energy to spend on reproduction. The story is also complex in studies that included females, which showed larger ovaries and oviducts with more cells present in infected *Litoria verreauxii alpina* (Brannelly et al., 2016), but a decrease in mature egg size and number in infected females of the same species (Brannelly et al., 2021). The differences in female reproductive response in these two studies were likely related to female age – young animals in the 2021 study may have prioritized pathogen defense, whereas the older animals used in the 2016 study may have been more sexually mature and therefore able to prioritize reproduction. Whether *Bsal* exhibits similar effects on reproductive fitness is not known. To date, no studies have examined reproduction and recruitment in salamanders in the face of *Bsal* exposure.

In this study, we explored gametogenesis and reproductive behaviors in the eastern newt (*Notophthalmus viridescens*) in response to *Bsal* exposure. The eastern newt was chosen for this study because it is abundant, and has been shown in previous experiments to reliably contract *Bsal* infection and survive at low exposure doses (Carter et al., 2021; Tompros, Dean, et al., 2022). Males and females can be distinguished visually during the breeding season, and mating behavior has been observed previously in laboratory environments. We measured reproductive potential and effort in newts exposed to *Bsal*, compared to unexposed controls. In males, we measured testicular size relative to body mass, seminiferous tubule density, germinal epithelial depth, and proportion of spermatogenesis stages as a proxy for reproductive effort. In females we measured mass of the gonads, size of the largest eggs, and proportion of developed eggs. We also paired males and females for observations of breeding behavior and to document any successful reproduction. Based on previous studies, we hypothesized that *Bsal*-exposed newts would exhibit terminal investment, evidenced by increased measures of gametogenesis and increased offspring production compared to unexposed newts. The effects of disease on reproduction are not often studied in amphibians, and this is the first to examine these effects in male and female salamanders.

Methods

Animal Husbandry

One hundred forty *Notophthalmus viridescens* (eastern newt, NOVI) adults were captured in the wild in Tennessee, US and transported to the Johnson Animal Research and Teaching Unit at University of Tennessee, Knoxville. Collection occurred early in the breeding season (mid-February) to ensure that animals were reproductively active, and they were sexed based on external characteristics. Males were identified by their keeled tails, wider back legs, prominent cloaca, and the presence of nuptial pads (Rucker et al., 2021). They were housed individually in 1L containers filled with approximately 300mL of dechlorinated water and a PVC cover object. We fed animals bloodworms (2% of their body mass every 3 days) and replaced each animal's container, cover object, and water every 3 d. Animals were kept in environmental chambers on a 12-h light, 12-h dark cycle, at >90% humidity. Temperature was gradually raised by 2C per day from ambient temperature to 30C and then held for 10 d to clear any pre-existing *Bd* infections (Chatfield & Richards-Zawacki, 2011). The temperature was then lowered by 2C per day to 14C, the approximate optimal growth temperature of *Bsal* (Martel et al., 2013), and the animals were acclimated to this temperature for 2 weeks.

Bsal Growth and Exposure

Bsal was isolated from a fire salamander (*Salamandra salamandra*) in the Netherlands (isolate AMFP13/1,(Martel et al., 2013)) and cultures were maintained at the University of Tennessee Center for Wildlife Health laboratory. *Bsal* zoospores used for exposure were harvested from tryptone gelatin hydrolysate (TGhL) agar plates after 6 d of growth. Plates were flooded with 7 mL of autoclaved dechlorinated water and filtered with a 20-µm sieve to isolate zoospores. Zoospores were enumerated using a hemocytometer and verified using flow cytometry (Carter et al., 2020).

Animals were randomly assigned to either Experiment 1 (Individual) or Experiment 2 (Breeding). All animals were also randomly assigned to one of 3 exposure groups: 1) none 2) single or 3) double. Those in the unexposed group were given a mock exposure at days 0 and 30, those in the single exposure group were exposed to *Bsal* at day 0 and a mock exposure at day 30,

and those in the double exposure group were exposed to *Bsal* at day 0 and 30. Animals were exposed in 100-mL plastic cylindrical containers with 9 mL of autoclaved dechlorinated water and 1 mL of the *Bsal* zoospore dose (5000 zoospores). Mock exposures were identical but consisted of 10 mL of autoclaved dechlorinated water. After 24 h, we removed newts from the inoculation tubes and placed them back into their tubs.

Experiment 1: Individual Reproductive Fitness

Animals within each exposure group were randomly assigned to either an "acute" or "chronic" infection group, where members of the acute group were euthanized 30 d following last *Bsal* exposure, and members of the chronic group were euthanized 60 d following last *Bsal* exposure. Unexposed animals were also assigned to these euthanasia dates. Treatment groups and euthanasia timing are presented in Table 3.1 (all tables and figures are shown in an appendix at the end of each chapter).

Starting on day 4 following *Bsal* exposure, animals were swabbed for *Bsal* qPCR every 6 d, using standardized swabbing protocols for *Bd* and *Bsal* (Blooi et al., 2013). Quantitative PCR for *Bsal* was run on each necropsy swab and other selected swabs following methods described in Carter et al (2020). Swabs continued on this schedule until each animal had three consecutive negative swabs. We monitored twice daily for signs of *Bsal* chytridiomycosis, including lethargy, focal lesions, ulcerations, increased skin sloughing, hemorrhage, anorexia, and loss of righting response (Carter et al., 2020; Martel et al., 2013).

On the prescribed euthanasia date, animals were euthanized using benzocaine solution. The whole body was then preserved in an individual whirl pak bag in 10% neutral buffered formalin. Following formalin fixation, whole body measurements were acquired, and gonads were dissected and weighed, then placed in individual cassettes for histology processing. Both left and right gonads were examined for each animal. Samples were embedded in paraffin and 5mm sections at mid-gonad depth were affixed to glass slides and stained with hematoxylin and eosin. All measurements were made using Excelis Accu-Scope software.

In males, the total area of the histological sections, the density of seminiferous tubules, the maximum germinal epithelium depth in the three largest seminiferous tubules per gonad were

measured. Each field of view containing the largest seminiferous tubules was used to quantify spermatogenesis stages (Figure 3.1). In amphibians, cells occur in spermatic cysts and each cyst only has one spermatogenesis stage, thus counts were by cyst rather than by individual cell.

In females, the size of the three largest eggs within each ovary were measured, and the proportion of eggs in each stage of development were counted. These were recorded as pre-vitellogenesis, vitellogenesis, and post-vitellogenesis, with guidance from Pewhorn et al. (2016) and Rodgveller (2018).

Experiment 2: Breeding Pairs

Animals in the breeding study were randomly assigned to pairs so that there were five of each pair type as follows: 1) Unexposed male/unexposed female 2) Unexposed male/single exposure female 3) Unexposed male/double exposure female 4) Single exposure male/unexposed female 5) double exposure male/unexposed female 6) single exposure male/single exposure female 7) double exposure male/double exposure female (Table 3.2). Following the second round of *Bsal* exposures, each male and female pair was housed in a 1L container filled with approximately 600mL of dechlorinated water and a plastic aquarium plant. Monitoring, swabbing, feeding, and cleaning procedures were the same as previously stated. Containers were also checked twice daily for spermatophores and eggs. Pairs were housed together for a total of 60 days, at which point the males were removed and euthanized. Females were euthanized 30 days later, after continued monitoring for egg-laying. The same necropsy and histopathology procedures were followed as for the individual study.

Statistical Analysis

All statistical analyses were performed in RStudio version 2022.12.0. For each gonad/gametogenesis measure, we conducted a two-way analysis of variance (ANOVA) for continuous dependent variables, where the independent variables were number of exposures, euthanasia date, and the interaction between number of exposures and euthanasia date. When the ANOVA was significant ($\alpha = 0.1$), Tukey Honestly Significant Difference tests were performed to evaluate pairwise differences in gonadal measurements. To assess oogenesis stages of the cells, we compared the proportion of total eggs counted that showed egg development

(vitellogenic and post-vitellogenic) per individual using a generalized linear model (GLM) with a binomial distribution, where the fixed effects were number of exposures, survival time, and the interaction of the two. As the overwhelming majority of spermatogenesis cell clusters in the males were spermatogonia, statistical analysis was only performed on these counts. Cell clusters were standardized to the total spermatogonia cluster count per 100 clusters, and were compared using a GLM with a negative binomial distribution, where the fixed effects were number of exposures, survival time, and the interaction term. In the breeding study, the same analyses were performed, where the independent variables were the exposure status of the female, the exposure status of the male, and the interaction between the exposures.

Results

Bsal Infection

All unexposed control animals remained *Bsal* negative throughout the study. None of the exposed animals in the individual study were *Bsal* positive for more than one swab following exposure. One double exposure female and control male in the breeding study that were paired together developed clinical chytridiomycosis and died, on day 73 and day 82 respectively.

Oogenesis

Statistical analyses of oogenesis measures are summarized in Table 3.3. Histologic examination of the ovaries revealed that the proportion of eggs with yolk development (Figure 3.2) was low overall in these animals (mean = 0.12, median = 0.10), with most eggs in the pre-vitellogenic stage. We fit a generalized linear model with binomial distribution to predict the proportion of eggs with yolk development based on *Bsal* exposure and euthanasia day. The model including the interaction between exposure and euthanasia day was a better fit than the model without the interaction term, based on a likelihood ratio test ($p < 0.001$). This model's intercept, corresponding to no *Bsal* exposure and death at day 30, is at -3.28. The proportion of eggs with yolk development was significantly higher overall in females that were exposed to *Bsal* once (beta = 0.90, $p = 0.007$) and in those that were exposed twice (beta = 1.62, $p < 0.001$). However, since the interaction term was significant at all levels, the effect of *Bsal* exposure on yolk development was different depending on how long the animal was alive post-exposure.

Yolk development was actually decreased at 60 days in both the single-exposure (beta = -1.39, $p < 0.001$) and double-exposure animals (beta = -1.69, $p < 0.001$). At 90 days, yolk development was greater in the double-exposure group (beta = 1.70, $p < 0.001$). Neither number of exposures nor survival time had a significant effect on the size of the largest eggs.

There was a trend towards lower relative ovary mass in animals that lived longer – ovaries of animals that were euthanized on day 30 were on average 1.17 times larger than those euthanized on day 60, and 1.48 times larger than those euthanized on day 90 (Figure 3.3). This was significant based on the ANOVA ($p = 0.02$). Tukey HSD revealed a significant difference in mass at day 90 compared to day 30 (diff = -0.01, $p = 0.047$), but no significant difference between day 60 and day 30 (diff = -0.005, $p = 0.46$) or day 90 and day 60 (diff = -0.006, $p = 0.28$). This effect was not different between exposure groups.

Spermatogenesis

Statistical analyses of spermatogenesis measures are summarized in Table 3.4. Testicular mass relative to body mass was higher in animals that lived 60 days (mean = 0.031, sd = 0.008) and 90 days (mean = 0.025, sd = 0.009) than those that lived 30 days (mean = 0.022, sd = 0.006) Based on the ANOVA, the effect of later euthanasia day on relative testicular mass was statistically significant overall ($p = 0.043$), whereas the effect of exposure was not ($p = 0.286$). Tukey HSD revealed that only the difference between animals that lived 60 days and those that lived 30 days was significant ($p = 0.089$)

Histologic examination revealed that the testes were dense and quiescent in most cases, with few animals exhibiting active spermatogenesis beyond spermatocyte formation, and only one animal (in the double exposure group, euthanized day 90) had mature spermatozoa. Germinal epithelial depth in millimeters was higher overall in the double-exposure animals (mean = 0.147, sd = 0.023) compared to the single exposure (mean = 0.11, sd = 0.04) and unexposed (mean = 0.12, sd = 0.04) animals (Figure 3.5). This effect was not dependent on survival time, as the interaction term was not significant and was therefore excluded from the model. The effect of exposure on germinal epithelial depth was significant in the ANOVA ($p = 0.088$) and Tukey HSD revealed that the difference in germinal epithelial depth between the

single and double exposure males was significant (p = 0.089) while the differences between the controls and the single-exposed animals (p = 0.79) and the control and the double-exposed animals (p = 0.19) were not. There was no significant effect of either number of exposures or survival time on testicular area, density of seminiferous tubules, or proportion of spermatogonia cell clusters.

Breeding Study

No spermatophores or eggs were found in the breeding animal containers during the study period. Statistical analyses of oogenesis in the breeding females are summarized in Table 3.5. Females that were exposed to Bsal twice and paired with a male that was also exposed twice had a lower proportion of eggs with yolk development compared to the females in the control/control pairs (Figure 3.6). This effect was significant at $\alpha = 0.1$ ($z = -1.702$, $p = 0.089$). Statistical analyses of spermatogenesis in the breeding males are summarized in Table 3.6. Total testicular area was lower in males exposed to *Bsal* once than in the control males (Tukey HSD p-value = 0.03), regardless of the number of exposures of the female of the pair (Figure 3.7), but this difference was not seen in the double-exposed males. Proportion of spermatogonia cells was lower in males paired with double-exposed females ($z = -1.893$, $p = 0.058$), indicating higher sperm maturation regardless of the exposure status of the male (Figure 3.8). Similar to the results seen in the individual study, most animals had minimal active gametogenesis.

Discussion

Our study suggests that *Bsal* exposure does affect gametogenesis in both male and female eastern newts. The measure that indicated the greatest effect of exposure was yolk development in the females. A tendency towards higher proportions of developed eggs in *Bd* infected females has been seen previously (Brannelly et al., 2016) in sexually mature animals. Here, this effect was greatest in animals that were exposed to *Bsal* twice and lived to 90 days. This suggests that additional *Bsal* exposures may induce greater reproductive output over time, which has biological significance as animals are unlikely to only be exposed to the pathogen once in the wild.

There was also evidence of terminal investment seen in the males, which exhibited greater germinal epithelial depth overall in the double-exposure group. A similar effect was seen on the germinal epithelium in male *Pseudophryne corroboree* (Brannelly et al., 2016) and has been suggested as an indicator of increased reproductive effort. This may indicate that additional *Bsal* exposures had a small positive effect on testicular growth. We did not detect exposure-based differences on the most direct measures of spermatogenesis – seminiferous tubule size and spermatogenesis stage of the cell clusters. However, testes overall were quiescent, and detecting differences between groups was difficult due to so few animals exhibiting active spermatogenesis.

The overall trends that were seen based solely on euthanasia timing may reflect natural progressions in gonad development and gametogenesis over time during the breeding season, rather than any effect of the study. Notably the females had a decrease in relative ovary size over time, but an increase in proportion of mature oocytes. This would seem to indicate that while oocyte development continued over time, initiation of oogenesis may have been halted. Additionally, there was some evidence of atresia and absorption of mature oocytes seen in animals where these were present, perhaps due to a lack of available males to initiate the fertilization and egg-laying process. Meanwhile males exhibited testicular growth over time, regardless of exposure status. These parameters are important to note as it may indicate that a longer study period may have provided a greater opportunity to evaluate gametogenesis metrics across exposure groups, as the animals became more reproductively active later into the breeding season.

The results from the breeding study in some ways contradict what was seen in the individually-housed animals. While double-exposed females housed individually had higher proportions of eggs with yolk development, the opposite effect was seen when they were housed with double-exposed males. Meanwhile the double-exposed males in the breeding study were actually more reproductively active than the control males, with a lower proportion of cells in the earliest stages of spermatogenesis. It is possible that behaviors were missed since the containers were not monitored 24/7, and females may in fact have laid eggs and then consumed them. The latter would perhaps explain the lower proportion of eggs with yolk development in the double-

exposed females, as they may have laid the developed eggs. In addition to the male exposure variable, the females in the breeding study were kept alive longer after exposure than those in the individual study, so direct comparisons between the two groups cannot be made. The lack of reproductive behaviors and reproductive output in the control groups makes it difficult to draw any conclusions from the breeding study, and future studies on the effect of *Bsal* exposure on breeding should follow a study design that more closely resembles conditions in the wild, ideally with animals in groups instead of pairs and in a more natural environment.

Overall, the animals in this study appeared to be reproductively quiescent. Neither males nor females were producing significant numbers of mature gametes, which is in contrast to previous passing observations of mature oocytes and spermatozoa on histopathology of numerous individuals of this species during other studies (see Chapter 2) where they were kept under similar environmental conditions. Since these animals were wild-caught, sexual maturity was assumed based on size and appearance, as their ages were unknown. At the time of collection, these animals were preparing for the breeding season and were distinguishable based on physical characteristics, and thus were assumed to be reproductively active. However, the amount of time spent under laboratory conditions may have dampened reproductive activity. In particular, heat treatment to clear any existing *Bd* infection would have brought their body temperatures above the ideal temperature for breeding, which may have altered reproductive status. This was seen externally as well, as the males and females became more difficult to distinguish from each other late in the study period.

An additional limitation of the study in evaluating the effect of *Bsal* on reproductive fitness is that the animals cleared their infections early on in the course of the study, whereas animals in previous studies were infected for a longer period of time (Brannelly et al., 2016, 2021; Chatfield et al., 2013). The *Bsal* dose used in this study was intentionally low to prevent death, as this species is highly susceptible and we were interested in outcomes for survivors, but this may have led to minimal effort required to resist infection. A *Bsal* dose that causes infection in eastern newts without leading to significant mortalities has not been established. Notably, the two animals that did maintain infections during the study did also succumb to disease.

Our study demonstrated the trend towards terminal investment that has been seen in other species in response to chytrid exposure. While an increase in reproductive output in the face of disease is beneficial in the short term for the individual and for population maintenance, it could also dampen the selection pressure for animals that are disease-resistant, therefore minimizing the development of population-level resistance to disease. Therefore, terminal investment should not be assumed to be of long-term benefit for the species, though it may bolster populations in the short term. We have shown that eastern newts that are exposed to *Bsal* even without developing severe infections experience some impacts on their reproductive potential, using gonadal characteristics and gametogenesis as a proxy. However, additional measures such as gamete viability, embryo development, and offspring survival are also vital contributors to the overall reproductive success of a population and remain as further avenues for exploration in understanding the overall impacts that *Bsal* has on susceptible species populations.

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Appendices

Appendix A: Tables

Table 3.1 Sample sizes of each of the treatment groups in Experiment 1: Individual Reproductive Fitness. Animals were randomly assigned to an exposure group and a euthanasia timepoint group.

	30 Days	**60 Days**	**90 Days**
Unexposed	5 male 5 female	5 male 5 female	5 male 5 female
Single Exposure	5 male 5 female	5 male 5 female	
Double Exposure		5 male 5 female	5 male 5 female

Table 3.2 Sample sizes of each of the treatment groups in Experiment 2: Breeding Study. Animals were randomly assigned to an exposure group, and then to a pair. The single exposure x single exposure group has one fewer pair than the others because one male was misidentified as a female.

	Males		
Females	**Unexposed**	**Single Exposure**	**Double Exposure**
Unexposed	5 pairs	5 pairs	5 pairs
Single Exposure	5 pairs	4 pairs	
Double Exposure	5 pairs		5 pairs

Table 3.3 Overview of the statistical analyses performed for female gonadal characteristics and oogenesis, summarizing the results and indicating how the dependent variable was affected by number of exposures and/or survival time. Bolded numbers indicate *p*-values <0.1.

Dependent variable	**Statistical Model**	**Predictors**	**Test statistic**	***p*-value**	**Effect Direction**
Female gonadal characteristics: significant effects					
Proportion of eggs with yolk development					
	GLM	Exposure	Z = 2.704 (single) Z = 4.345 (double)	**0.007** <**0.001**	Higher in animals that were exposed once. Higher in animals exposed twice
		Survival Time	Z = 5.545 (60 d) Z = 0.400 (90 d)	<**0.001** 0.689	Higher overall in animals that lived 60 days.
		Exposure * Survival Time	Z = -3.419 (single * 60d) Z = -3.884 (double* 60d)	<**0.001** <**0.001**	Lower in animals that were exposed once and lived 60 days. Lower in animals that were exposed twice and lived 60 days
Ovary mass relative to body mass	two-way ANOVA	Exposure	F = 0.203	0.818	
		Survival Time	F = 4.391	**0.023**	Lower relative ovary mass in animals that lived longer, not affected by exposure.
Female gonadal characteristics: no significant effects					
Size of largest eggs	two-way ANOVA	Exposure	F = 0.4560	0.639	
		Survival Time	F = 0.168	0.846	

Table 3.4 Overview of the statistical analyses performed for male gonadal characteristics and spermatogenesis, summarizing the results and indicating how the dependent variable was affected by number of exposures and/or survival time. Bolded numbers indicate *p*-values <0.1.

Dependent variable	**Statistical Model**	**Predictors**	**Test statistic**	***p*-value**	**Effect Direction**
Male gonadal characteristics: significant effects					
Testicular mass relative to body mass	Two-way ANOVA	Exposure	F = 1.266	0.303	
		Survival Time	F = 3.643	**0.043**	Higher in animals that lived longer, regardless of exposure.
		Exposure * Survival	F = 1.214	0.283	
Germinal epithelial depth	Two-way ANOVA	Exposure	F = 2.472	**0.088**	Germinal epithelial depth was greatest in the double-exposure males overall.
		Survival	F = 0.288	0.752	
Male gonadal characteristics: no significant effects					
Testicular area relative to body mass	Two-way ANOVA	Exposure	F = 2.052	0.153	
		Survival	F = 1.191	0.324	
Density of seminiferous tubules	Two-way ANOVA	Exposure	F = 1.989	0.162	
		Survival	F = 0.345	0.712	
Proportion of spermatogonia cell clusters	GLM (negative binomial)	Exposure: Single Double	Z = 0.187 0.096	 0.851 0.923	
		Survival: 60 d 90 d	 -0.303 -0.456	 0.742 0.648	

Table 3.5 Overview of the statistical analyses performed for female gonadal characteristics and oogenesis in animals in the breeding study, summarizing the results and indicating how the dependent variable was affected by number of exposures of the female and/or of the male they were paired with. Bolded numbers indicate *p*-values <0.1.

Dependent variable	**Statistical Model**	**Predictors**	**Test statistic**	***p*-value**	**Effect Direction**
Breeding female gonadal characteristics: significant effects					
Proportion of eggs with yolk development	Negative binomial	Exposure *Single* *Double*	 z= -0.820 z = 0.061	 0.412 0.951	
		Male Exposure *Single* *Double*	 z = -0.040 z = 0.341	 0.968 0.733	
		Exposure:Male Exposure *Single:Single* *Double:Double*	 z = 0.204 z = -1.702	 0.838 **0.089**	Females that were exposed to *Bsal* twice and paired with a male that was also exposed twice had a lower proportion of eggs with yolk development compared to controls.
Breeding female gonadal characteristics: no significant effects					
Ovary mass relative to body mass	Two-way ANOVA	Exposure	F = 0.274	0.762	
		Male exposure	F = 1.236	0.307	
Size of largest eggs	Two-way ANOVA	Exposure	F = 0.638	0.536	
		Male exposure	F = 0.166	0.848	

Table 3.6 Overview of the statistical analyses performed for male gonadal characteristics and spermatogenesis in animals in the breeding study, summarizing the results and indicating how the dependent variable was affected by number of exposures of the male and/or of the female they were paired with. Bolded numbers indicate *p*-values <0.1.

Dependent variable	**Statistical Model**	**Predictors**	**Test statistic**	***p*-value**	**Effect Direction**
Breeding male gonadal characteristics: significant effects					
Testicular area relative to body mass	Two-way ANOVA	Male exposure	F = 3.42	**0.049**	Total testicular area was lower in males exposed to *Bsal* once than in the control males (Tukey HSD p-value = 0.03).
		Female Exposure	F = 0.88	0.426	
Proportion of spermatogonia cell clusters	Negative binomial	Male exposure *Single* *Double*	 z = 0.093 z = -0.30	 0.926 0.764	
		Female exposure *Single* *Double*	 z = 0.341 z = -1.893	 0.733 **0.058**	Proportion of spermatogonia cells was lower in males paired with double-exposed females, indicating higher sperm maturation.
Breeding male gonadal characteristics: no significant effects					
Testicular mass relative to body mass	Two-way ANOVA	Male exposure	F = 0.130	0.878	
		Female exposure	F = 0.917	0.412	
Germinal epithelial depth	Two-way ANOVA	Male exposure	F = 1.880	0.173	
		Female exposure	F = 0.921	0.411	

Appendix B: Figures

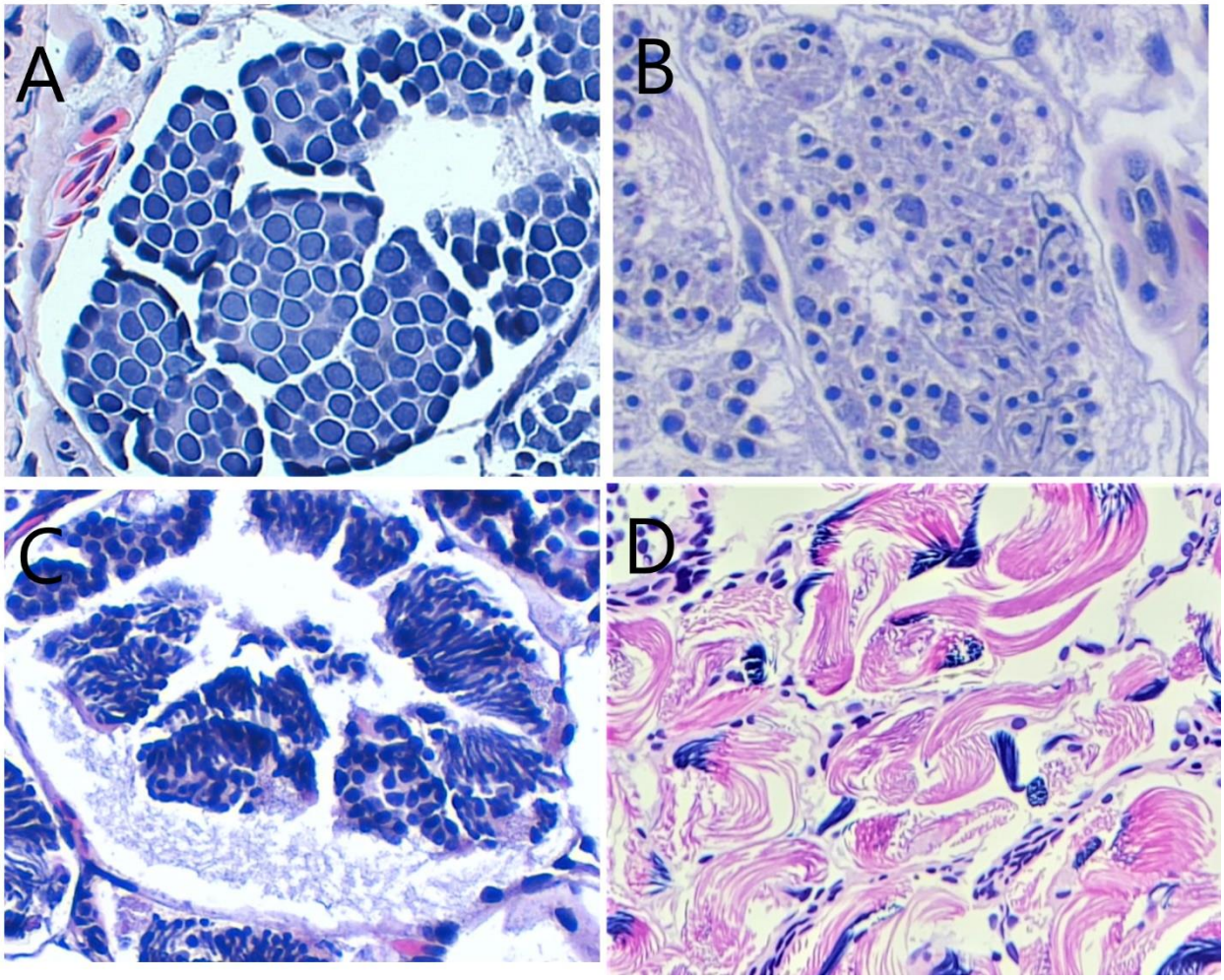

Figure 3.1 Stages of spermatogenesis. A) Spermatogonia B) Spermatocysts C) Spermatids D) Spermatozoa. All images are at 10x magnification.

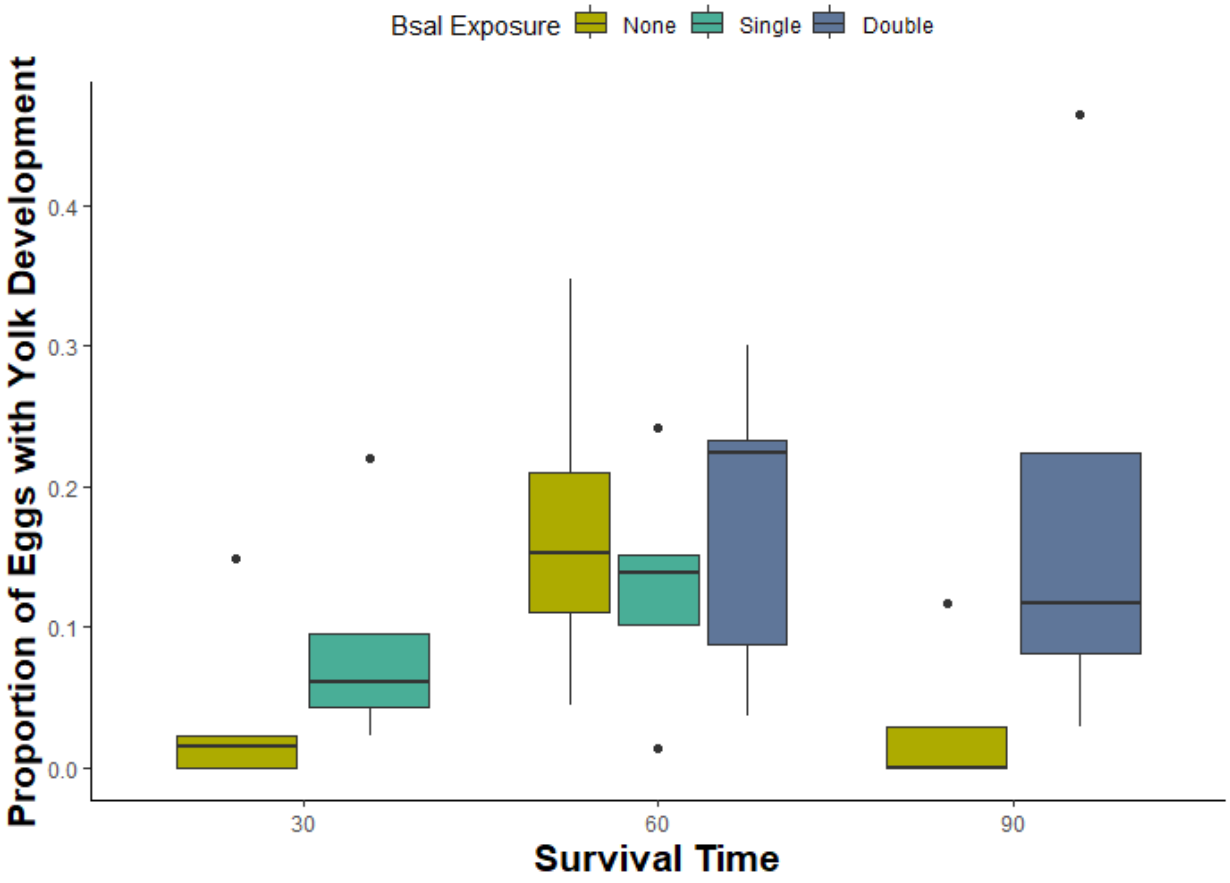

Figure 3.2 Oogenesis comparisons between *Bsal*-unexposed, single exposed, and double exposed female *Notophthalmus viridescens*, that were euthanized at 30, 60, or 90 days after the first exposure day. Oogenesis stage is presented as eggs with yolk development (vitellogenic and mature stages) as a proportion of the total eggs counted for each individual.

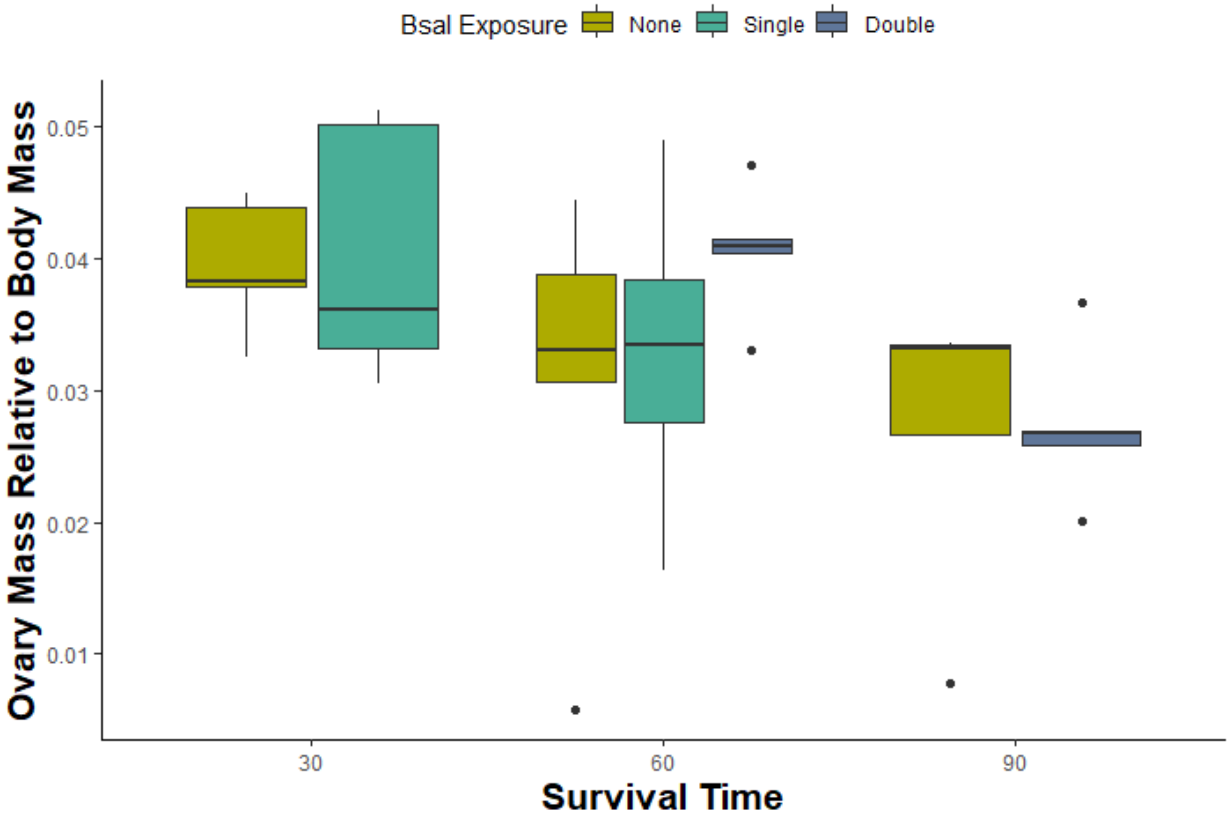

Figure 3.3 Gonad comparison between *Bsal*-unexposed, single exposed, and double exposed female *Notophthalmus viridescens*, that were euthanized at 30, 60, or 90 days after the first exposure day. Ovary mass is presented as a proportion of the body mass of the individual.

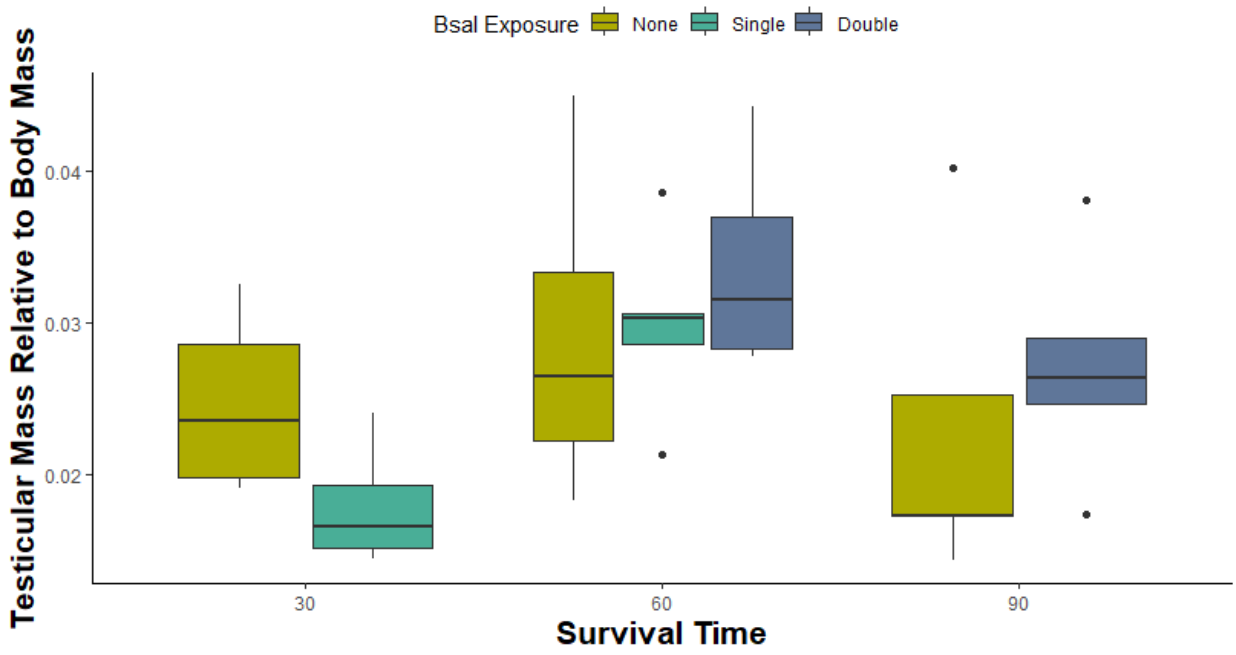

Figure 3.4 Gonad comparison between *Bsal*-unexposed, single exposed, and double exposed male *Notophthalmus viridescens*, that were euthanized at 30, 60, or 90 days after the first exposure day. Testicular mass is presented as a proportion of the body mass of the individual.

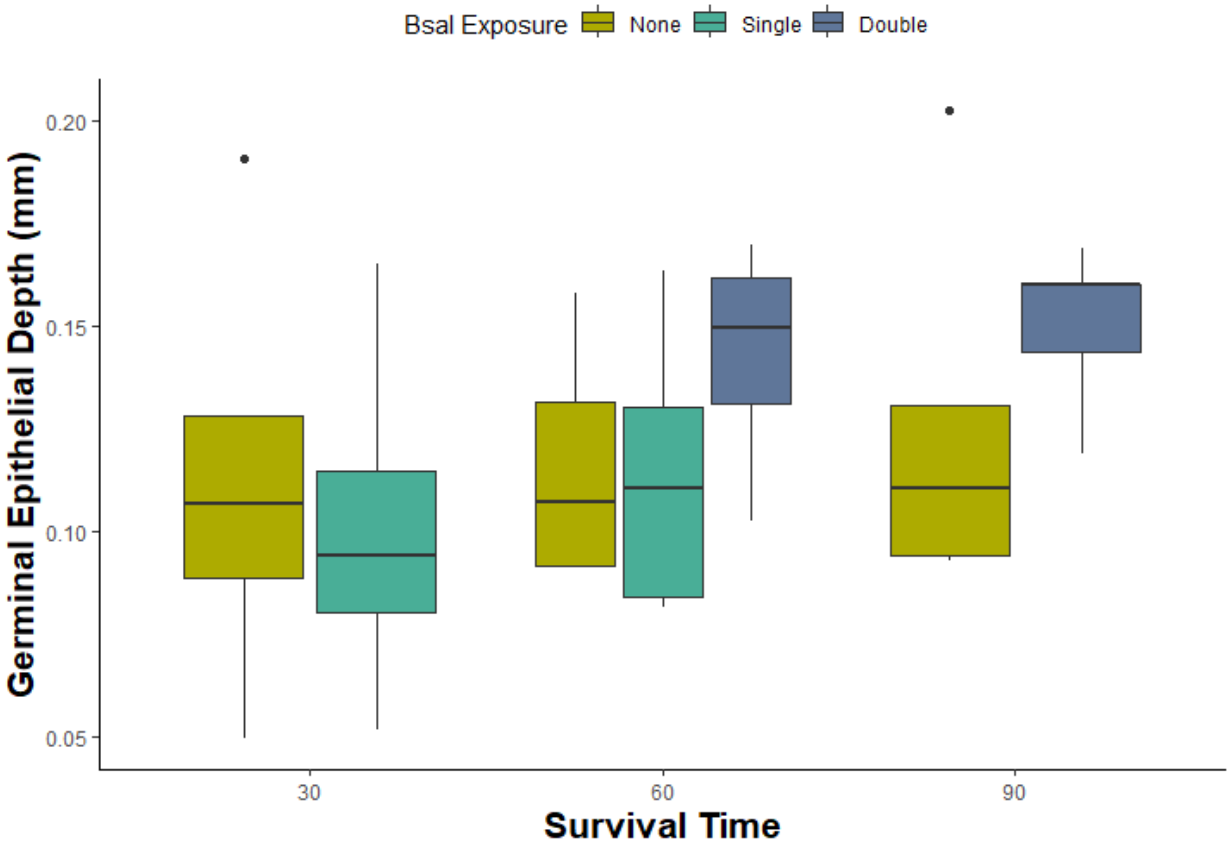

Figure 3.5 Gonad comparison between *Bsal*-unexposed, single exposed, and double exposed male *Notophthalmus viridescens*, that were euthanized at 30, 60, or 90 days after the first exposure day. Germinal epithelial depth was averaged across the six largest seminiferous tubules for each individual.

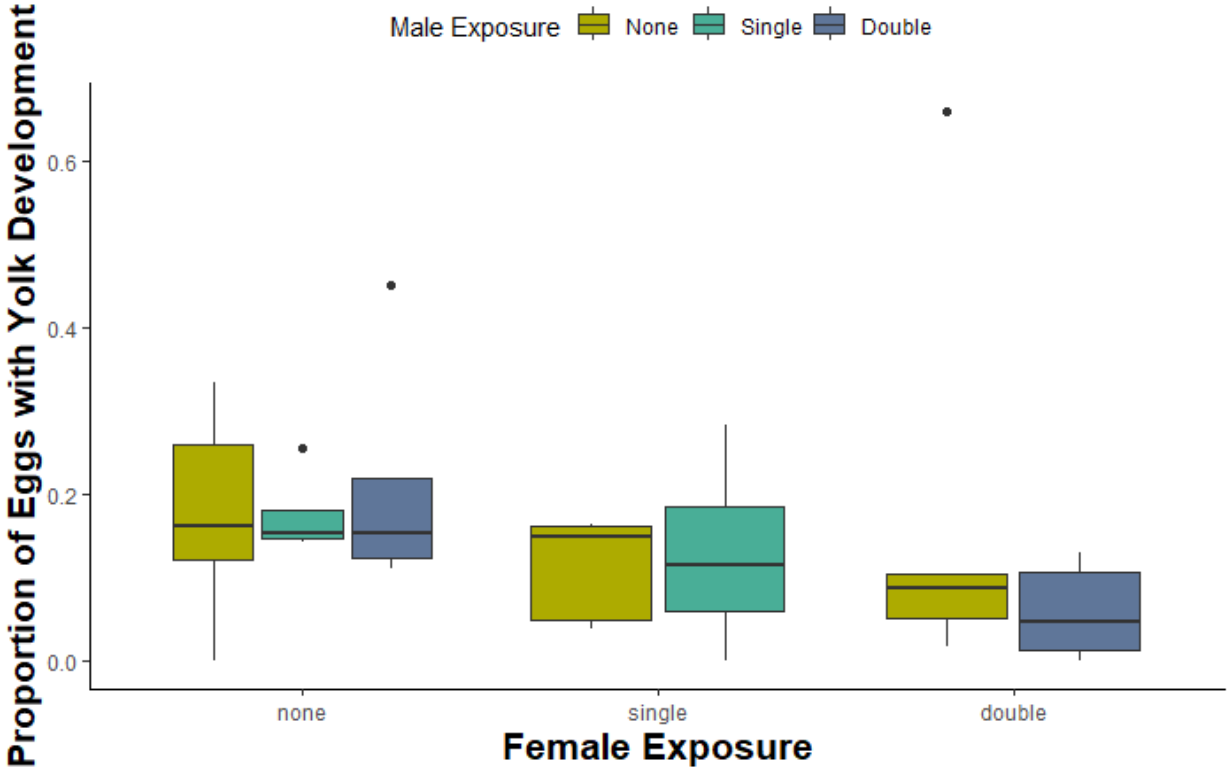

Figure 3.6 Oogenesis comparisons between *Bsal*-unexposed, single exposed, and double exposed female *Notophthalmus viridescens*, that were paired with unexposed, single exposed, or double exposed males for 60 days. Oogenesis stage is presented as eggs with yolk development (vitellogenic and mature stages) as a proportion of the total eggs counted for each individual.

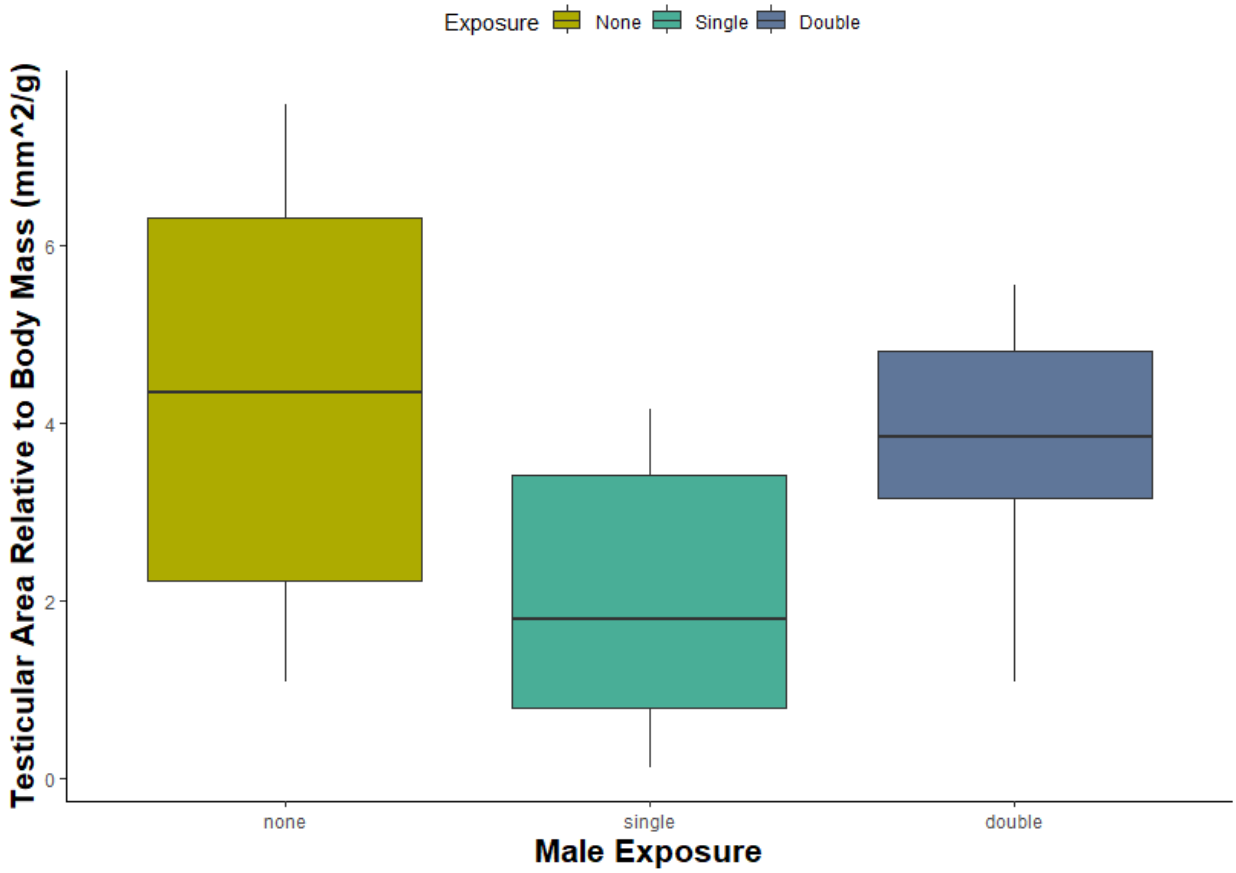

Figure 3.7 Gonad comparison between *Bsal*-unexposed, single exposed, and double exposed male *Notophthalmus viridescens*, that were paired with unexposed, single exposed, or double exposed females. Testicular area on histologic examination is presented relative to overall body mass.

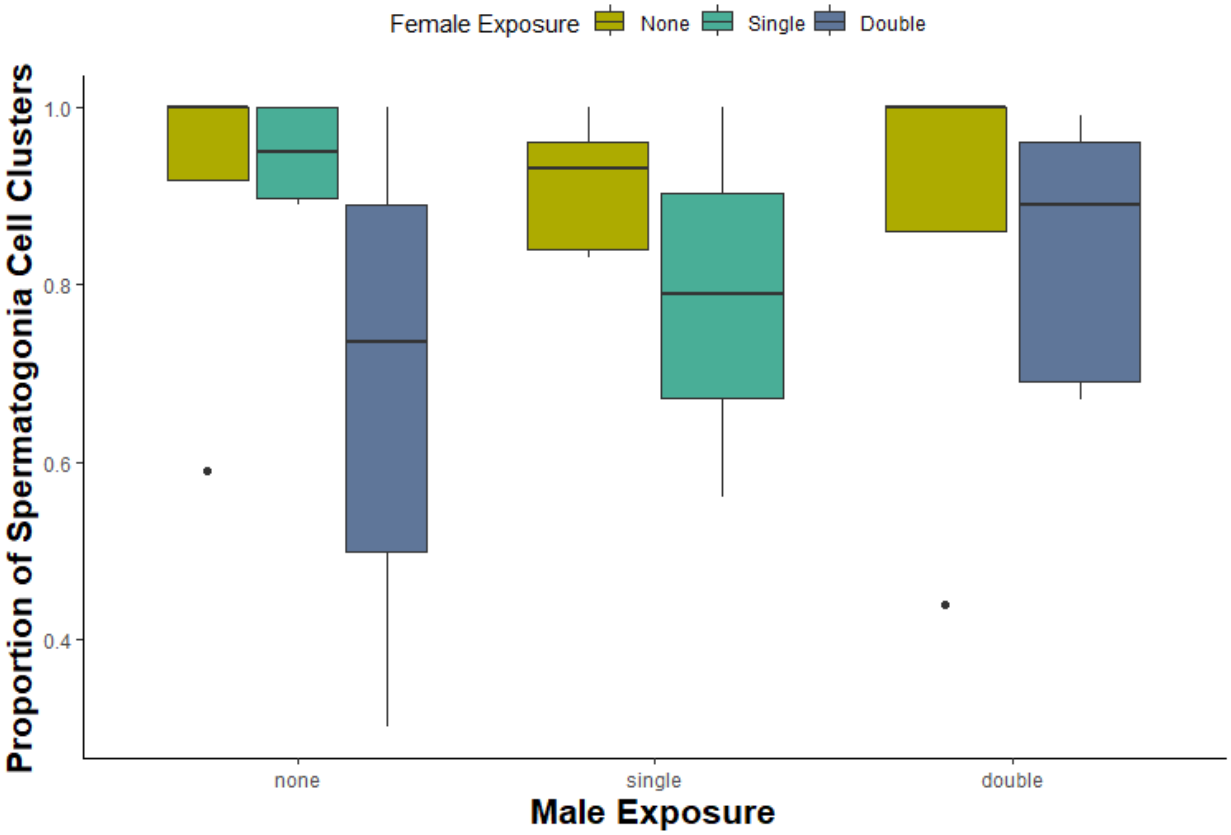

Figure 3.8 Spermatogenesis comparisons between *Bsal*-unexposed, single exposed, and double exposed male *Notophthalmus viridescens*, that were paired with unexposed, single exposed, or double exposed females for 60 days. Spermatogenesis stage is presented as spermatogonia (the earliest stage) cell clusters as proportion of the total cell clusters.

Conclusion

Chytridiomycosis is a devastating disease for amphibian populations worldwide, and has the potential to cause more population declines and even extinctions as it spreads (Scheele et al., 2019). Both fungal pathogens that cause chytridiomycosis, *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*), have been the subjects of intensive research to inform mitigation efforts around the globe. The goals of the studies presented here were to evaluate potential treatment options for chytridiomycosis, and to investigate aspects of the pathogenesis of *Bsal* in particular that were less well-understood – namely the role of secondary bacterial infiltration in morbidity and mortality, and the impact of exposure on reproductive fitness.

Terbinafine has been used as a topical treatment for chytridiomycosis (Guzman et al., 2022), however the requirement for multiple doses is problematic when considering the treatment of wildlife. Subcutaneous implants impregnated with the drug have been used in pharmacokinetic studies in cottonmouths and hellbenders (Hardman et al., 2021; Kane et al., 2017), and we were interested in exploring the use of these implants through an intracoelomic route to treat *Bd* in greater sirens. While the efficacy of this treatment for *Bd* remains unclear, the intracoelomic injection route appears to be a safe method for administering the implants. Future studies investigating the use of intracoelomic implants in other amphibian species, with different drugs, and aimed at identifying effective doses, are warranted. The use of these implants may provide a practical method for long-term drug delivery in free-ranging wildlife and in headstart programs, even outside of the chytridiomycosis disease system.

Our next goal was to investigate the use of probiotic treatments to combat *Bsal* chytridiomycosis, and to evaluate the contribution of bacterial infections to morbidity and mortality in the course of the disease. Probiotics have shown promise in laboratory settings in the treatment of *Bd* (Harris et al., 2009), but there is also evidence of septicemia contributing to *Bsal* pathogenesis (Bletz et al., 2018). Despite a trend towards lower *Bsal* loads in animals exposed to *Pseudomonas fluorescens* after low-dose *Bsal* exposure, *P. fluorescens* as administered in this study was not an effective probiotic against *Bsal* chytridiomycosis. Conversely, exposure to *P. fluorescens* may lead to increased infiltration of bacteria in the blood and organs in *Bsal*-exposed animals, with the greatest increase occurring when skin lesions are already present. Future

studies should explore the use of different organisms or even a combination of organisms as probiotic treatments, keeping in mind their usage may be restricted to captive settings, where dosing and administration frequency can be more strictly controlled. Meanwhile, the findings in this study indicate that some caution should be exercised when exposing immunocompromised animals, especially those with skin lesions, to bacteria.

Finally, we investigated the impact that *Bsal* exposure has on reproductive fitness in eastern newts during the breeding season. Previous studies involving *Bd* infection have shown an overall trend towards terminal investment (An & Waldman, 2016; Brannelly et al., 2016; Chatfield et al., 2013; Roznik et al., 2015) , particularly in animals that are otherwise healthy and have reached peak sexual maturity. Our study demonstrated a similar impact of *Bsal* exposure in eastern newts, even when they did not develop significant infections. Males and females both showed evidence of increased reproductive effort (increased germinal epithelial depth and increased egg yolk development, respectively) with *Bsal* exposure, particularly with multiple exposure events. Additional measures related to successful breeding and offspring viability remain as further avenues for exploration in understanding the overall impacts that *Bsal* has on susceptible species populations, beyond the acute infection phase.

Overall, treatment options for chytridiomycosis remain an area of necessary research, and the findings of these studies serve as a foundation for further efforts to optimize treatment methods, particularly for free-ranging wild amphibians. A deeper understanding of the acute pathogenesis of the disease is important not only for developing those treatments, but also for mitigating unintended consequences, as we saw in the use of *P. fluorescens* as a probiotic. Meanwhile, lasting impacts of the disease at the individual level will continue to have population-level implications if, as demonstrated here, exposed animals tend towards increased reproductive effort in an attempt to produce offspring before succumbing to disease. Whether this effect will serve to select for animals that are disease-resistant is beyond the scope of our investigation, but is an important factor to explore in understanding overall disease impacts on a population.

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Vita

Anastasia Towe moved from her hometown of Jacksonville, Florida in 2011 to attend the University of Pittsburgh, graduating with a Bachelor of Science in Biology and a Bachelor of Arts in English Writing in 2015. She worked as a small animal veterinary assistant before starting the Doctor of Veterinary Medicine degree program in 2016 at the University of Tennessee College of Veterinary Medicine. She began working in the Gray-Miller Amphibian Disease lab in 2017 and joined the Comparative and Experimental Medicine dual-degree program under the mentorship of Dr. Debra Miller in 2018. She completed her DVM in 2020 and continued to pursue her Doctor of Philosophy degree. Her research interests include the pathogenesis and treatment of emerging wildlife diseases, and particularly their impacts on reproductive health in wild populations. After graduation, she will begin her new position as an exotics and zoological medicine intern at Kansas State University.