ELUCIDATING THE PATHOGENESIS OF *BATRACHOCHYTRIUM SALAMANDRIVORANS* CHYTRIDIOMYCOSIS

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ABSTRACT

Amphibian species worldwide are facing severe threats to their biodiversity including infectious disease, climate change, and habitat destruction. With these increasing threats, there is a growing need to explore disease pathogenesis and understand amphibian health. One of the most important emerging infectious diseases of amphibians is caused by the fungal pathogen *Batrachochytrium salamandrivorans* (*Bsal*). *Bsal* was recently discovered and is of global concern due to its potential to cause high mortality in amphibians, primarily salamander species. We investigated disease pathogenesis using two newt species with known susceptibility to *Bsal* chytridiomycosis (*Notophthalmus viridescens* and *Taricha granulosa*) as models. *Taricha granulosa* were exposed to *Bsal* and allowed to reach various stages of disease progression. Blood was collected immediately postmortem for hematological and biochemical analysis as well as plasma protein electrophoresis. Histologic skin lesions associated with *Bsal* infection were quantified and graded for each animal. Electrolyte imbalances and dehydration due to epidermal damage were shown to likely be the primary cause of morbidity and mortality in diseased *T. granulosa*. In a separate study, *N. viridescens* were exposed to one of four *Bsal* zoospore doses at one of three environmental temperatures. Individuals exposed at 14°C had increased lesion counts compared to those exposed at 6°C. Individuals exposed at 22°C did not become infected. Also, increased lesion counts were associated with decreased survival rate, and the most common anatomic sites for lesions of any grade were the hindlimbs, cloacal region, and tail. Independent of *Bsal* investigations, protein electrophoresis was performed on plasma collected from one anuran and six urodelan species that were clinically normal. This study provided novel representative electrophoretograms for each species and lay groundwork for future studies to establish reference intervals in these as well as other amphibian species. Overall, the findings of my research provide insight into the pathogenesis of *Bsal* chytridiomycosis and provide evidence that plasma protein electrophoresis may be a useful diagnostic tool in amphibians. This information can be used to identify possible disease mitigation options for *Bsal* chytridiomycosis in captive settings, provide guidance for *Bsal* surveillance programs, and will be helpful in future amphibian health investigations.

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INTRODUCTION

We are currently in the process of a sixth mass extinction (Ceballos et al., 2020), with Amphibia being the vertebrate class experiencing the most severe declines (IUCN, 2022). Amphibians are extremely important as they are indicators of overall ecosystem health (Hocking & Babbitt, 2014; Welsh & Ollivier, 1998), are crucial for insect control, have critical roles in the food chain, are used as models in biomedical research, and have cultural importance to many societies (Hocking & Babbitt, 2014). Therefore, it is imperative that we work to decrease the threats amphibians are currently facing as well as obtain a better understanding of ways to monitor their health, in order to maintain their biodiversity.

The main threats to amphibians include climate change, habitat degradation and loss, pollution, overexploitation through their use as food and in the pet trade, introduction of invasive species, and emerging infectious diseases (IUCN, 2022). Among emerging infectious disease threats, the most significant is chytridiomycosis, which has been responsible for what has been referred to as the greatest documented loss of vertebrate biodiversity due to a pathogen (Scheele et al., 2019). There are two species of chytrid fungi which have been described as pathogenic to amphibians. These include the well-known species *Batrachochytrium dendrobatidis* (*Bd*), and the recently described species *Batrachochytrium salamandrivorans* (*Bsal*). Both infect the skin of amphibians, and either primarily cause proliferative (*Bd*) or ulcerative (*Bsal*) lesions. As the skin of amphibians is responsible for electrolyte balance, osmoregulation, as well as respiration, damage to this organ is detrimental (Wells, 2007).

While *Bd* has been documented on every continent that has amphibians, *Bsal* has not yet been identified in North America (Waddle et al., 2020). Due to suitable environmental conditions, a large number of susceptible host species, high prevalence of *Bsal* in internationally-traded amphibian species, and few trade regulations to reduce the risk of further global introductions, risk of invasion of *Bsal* into North America is high (Basanta et al., 2019; Carter et al., 2021; Grant et al., 2017; Richgels et al., 2016; Yap et al., 2015; Yap et al., 2017). Given that *Bsal* primarily infects urodelan species, and North America is a global hotspot for urodelan biodiversity (Yap et al., 2015), it is imperative that we understand how this pathogen causes morbidity and mortality in its hosts prior to its arrival. My dissertation fills three important gaps in our current knowledge surrounding *Bsal* and amphibian health: (1) the mechanisms of *Bsal* pathogenesis, (2) the effects of environmental temperature on disease progression, and (3) the use of protein electrophoresis as a diagnostic tool in amphibians.

Much work has been done to elucidate the pathogenesis of *Bd*; however, limited research on the mechanisms of *Bsal* exist. Therefore, Chapter I covers my investigation into the pathogenesis of *Bsal* chytridiomycosis through the use of anatomic and clinical pathology in *Bsal*-infected *Taricha granulosa*. By incorporating biochemical and hematological analyses along with plasma protein electrophoresis and histopathology, I investigated how *Bsal* causes morbidity and mortality in its hosts. Results of my research revealed the similarities and differences between *Bsal* and *Bd* chytridiomycosis.

Additionally, my results provide potential options for disease mitigation in *Bsal*-infected amphibians in captive settings.

In Chapter II, I evaluated how environmental temperature and *Bsal* zoospore dose affect disease development and progression in *Notophthalmus viridesens*. I also evaluated anatomic sites of predilection for *Bsal* lesion development. The results from this portion of my research provide an understanding of how environmental conditions can influence the rate of *Bsal* chytridiomycosis development. They also provide guidance for *Bsal* surveillance programs by identifying anatomic locations within the skin that display the highest lesion counts associated with *Bsal* chytridiomycosis. This information can be used to increase the chances of pathogen detection through skin swabbing for *Bsal* qPCR.

Finally, Chapter III reports efforts to begin to provide basic parameters for health in amphibians using protein electrophoresis. Protein electrophoresis has proven to be very useful in mammalian as well as other non-mammalian species (Cray, 2021). It can be used to assist in diagnosis of many disease processes and in evaluation of overall health status. Very limited studies have investigated its usefulness in amphibians. Therefore, we analyzed plasma protein electrophoretograms from clinically normal individuals of one anuran and six urodelan species. The results from this chapter in combination with results from Chapter I support the usefulness of this diagnostic tool in amphibians and provide groundwork for future investigations to develop reference intervals in these and other amphibian species which can be used to monitor amphibian health.

Overall, the findings reported in my dissertation significantly advance our understanding of the pathogenesis of *Bsal* chytridiomycosis and how environmental temperature affects disease progression. I also provide a new model for a multimodal diagnostic approach to the study of *Bsal* chytridiomycosis. Additionally, I provide evidence for the utility of a practical diagnostic tool which can be used to monitor amphibian health and assist in diagnosis of many disease processes, including *Bsal* chytridiomycosis.

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CHAPTER I: ELECTROLYTE IMBALANCES AND DEHYDRATION PLAY A KEY ROLE IN *BATRACHOCHYTRIUM SALAMANDRIVORANS* CHYTRIDIOMYCOSIS

Abstract

One of the most important emerging infectious diseases of amphibians is caused by the fungal pathogen *Batrachochytrium salamandrivorans* (*Bsal*). *Bsal* was recently discovered and is of global concern due to its potential to cause high mortality in amphibians, especially salamander species. To date, little has been reported on the pathophysiological effects of *Bsal*; however, studies of a similar fungus, *B. dendrobatidis* (*Bd*), have shown that electrolyte losses and immunosuppression likely play a key role in morbidity and mortality associated with this disease. The goal of this study was to investigate pathophysiological effects and immune responses associated with *Bsal* chytridiomycosis using 49 rough-skinned newts (*Taricha granulosa*) as the model species. *Taricha granulosa* were exposed to a 1 x 10^7 per 10 mL dose of *Bsal* zoospores and allowed to reach various stages of disease progression before being humanely euthanized. At the time of euthanasia, blood was collected for biochemical and hematological analyses as well as protein electrophoresis. Ten standardized body sections were histologically examined, in which *Bsal*-induced skin lesions were counted and graded on a scale of 1–5 based on severity. Results indicated that electrolyte imbalances and dehydration induced by damage to the epidermis play a major role in the pathogenesis of *Bsal* chytridiomycosis in this species. Additionally, *Bsal*-infected, clinically diseased *T. granulosa* exhibited a systemic inflammatory response identified through alterations in complete blood counts and protein electrophoretograms. Overall, these results provide integral information on the pathogenesis of this disease and highlight the differences and similarities between *Bsal* and *Bd* chytridiomycosis.

1. Introduction

Over the past five decades, chytridiomycosis has been responsible for the decline of >500 amphibian species, and the extinction of nearly 90 amphibian species, culminating in what has been described as the greatest documented loss of vertebrate biodiversity due to a pathogen (Scheele et al., 2019). The culprit behind the majority of these drastic declines and extinctions has been the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) which was first detected in the 1990s (Scheele et al., 2019). In 2013, a novel chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*), was first discovered in fire salamanders (*Salamandra salamandra*) and is causing mass die-offs of urodeles in multiple European countries (Lotters et al., 2020; Martel et al., 2013; Stegen et al., 2017). This pathogen mainly infects urodeles; however, it has also been shown to infect anuran species (Nguyen et al., 2017; Stegen et al., 2017; Towe et al., 2021).

Bsal presumably originated in Asia and is thought to have spread to Europe through the pet amphibian trade (Martel et al., 2014). This pathogen has not yet been detected in North America (Waddle et al., 2020). However, studies report that likelihood of *Bsal* invasion is high due to suitable environmental conditions, a large number of susceptible host species, high prevalence of *Bsal* in heavily-traded amphibian species, and few pet trade regulations to reduce the risk of further global introductions (Basanta et al., 2019; Carter et al., 2021; Grant et al., 2017; Richgels et al., 2016; Yap et al., 2015; Yap et al., 2017). Despite recognition of the severe threat that *Bsal* poses to global

amphibian biodiversity, we still know little about the mechanisms of its pathogenesis, limiting our ability to develop effective therapeutics to treat infected individuals.

While limited information has been reported on the pathophysiological effects of *Bsal*, studies of *Bd* have provided significant information on chytrid fungus pathophysiology. Studies of *Bd*, have shown that electrolyte imbalances occur in amphibians due to loss of osmoregulation through extensive skin pathology (Campbell et al., 2012; Marcum et al., 2010; Voyles et al., 2007; Voyles et al., 2009; Young et al., 2012). These electrolyte losses have been shown to lead to cardiac arrest which is likely the ultimate cause of mortality in *Bd*-infected individuals (Voyles et al., 2009).

Bd and *Bsal* are closely related to each other yet differ in the type of skin lesions they induce (Farrer et al., 2017; Martel et al., 2013). Amphibians infected with *Bd* typically develop proliferative epidermal lesions that result in thickening of the skin, whereas amphibians infected with *Bsal* develop erosive to ulcerative, necrotizing lesions that create holes in the skin (Martel et al., 2013; Van Rooij et al., 2015). Although the type of skin injury is different for each pathogen, it is hypothesized that the mechanisms by which each pathogen causes morbidity and mortality have similarities because they are both impacting skin function. However, differences such as potential secondary bacterial infections and dermal gland invasion in *Bsal*-infected amphibians are also suspected (Bletz, 2013; Ossiboff et al., 2019). Additionally, it has also been shown in previous studies that anorexia may play a role in *Bsal* pathogenesis (Carter et al., 2019). Here, I compared clinical presentation along with clinical and anatomic pathology changes in diseased versus control individuals by measuring blood cell counts, biochemical values, and plasma protein levels in combination with histologic examination of all organs. By combining histological and molecular techniques along with clinical presentation, my goal was to gain new understanding of how *Bsal* chytridiomycosis causes morbidity and mortality in susceptible hosts.

2. Methods

Ethics Statement

Husbandry as well as euthanasia procedures followed recommendations provided by the American Veterinary Medical Association and the Association of Zoos and Aquariums. Additionally, they were approved by the University of Tennessee Institutional Animal Care and Use Committee (protocol #2623). *Taricha granulosa* that reached euthanasia endpoints were humanely euthanized via transdermal exposure to benzocaine hydrochloride.

Animals

A total of 49 *Taricha granulosa* (rough-skinned newts) were wild-caught by California Department of Fish and Wildlife and shipped to the University of Tennessee. *Taricha granulosa* were used due to their known susceptibility to *Bsal* (Martel et al., 2014) and their relatively large size which allowed a sufficient amount of blood to be collected from each individual. Upon arrival to the laboratory, individuals were each given an individual ID (“TW1”-“TW49”). We confirmed that all *T. granulosa* were *Bd* negative by using

quantitative polymerase chain reaction (qPCR) methods similar to those described by Boyle et al. (2004). All animals were then heat-treated for 10 days at 30°C to clear any potential pre-existing *Bd* infections which may have not been detected by qPCR (Bletz, 2013; Chatfield & Richards-Zawacki, 2011). Co-infection with *Bd* can influence disease outcomes in *Bsal* chytridiomycosis (Longo et al., 2019); however, previous infection and clearance of *Bd* does not seem to have an effect on susceptibility to *Bsal* in other urodelan species (Longo et al., 2019). We again confirmed that all *T. granulosa* were *Bd* negative prior to the beginning of the trial by testing skin swabs using qPCR. The pre-trial swabs as well as all skin swabs performed throughout the study consisted of 10 swipes along the ventrum and 5 swipes on the bottom of each foot. After heat treatment, *T. granulosa* were acclimated for two weeks to a temperature of 14°C, which has been found to be the optimal temperature for *Bsal* growth and infection of urodelan species (Carter et al., 2021; Martel et al., 2013). Animals were individually housed in an environmental growth chamber (Conviron, Winnipeg, Canada) and temperature was maintained at 14°C throughout the entire study. The environmental growth chamber also provided light/dark cycles reflecting the corresponding season associated with the environmental temperature (spring/fall = 14 hours dark). Individual housing consisted of sterilized 710-cm^3 containers that included a moist paper towel and PVC cover object. All containers, paper towels, and cover objects were replaced with new ones every three days. Diet consisted of medium-sized crickets (amount based on 4% of each animal's body weight measured at the beginning of study) given at each water change and food consumption was noted at the following water change.

Experimental Design

The 49 *T. granulosa* were randomly assigned to either a control (n=8) or *Bsal*-exposed (n=41) treatment group. Animals were monitored twice daily for signs of chytridiomycosis. Detailed notes regarding lesion coverage, skin sloughing, and degree of lethargy were taken and graded at each of these timepoints (skin sloughing and lesion coverage: 0 = none; 1 = < 25% body coverage, 2 = 25–50% body coverage, 3 = 51–75% body coverage, 4 = >75% body coverage; Lethargy grade: 0 = none; 1 = mild; 2 = moderate; 3 = severe). Examples of skin sloughing (Figure 1.1), hemorrhage (Figure 1.2), and multifocal epidermal ulcerations (Figure 1.3) are provided in the Appendix. Those individuals who reached a pre-determined euthanasia endpoint (loss of righting ability), were humanely euthanized through transdermal administration of benzocaine. Others were euthanized at various stage of disease progression or at the end of the study (60 days). *Bsal*-exposed *T. granulosa* were classified as "clinically diseased" if they exhibited evidence of moderate to severe lethargy (loss of righting reflex) at the time of euthanasia. Control *T. granulosa* as well as *T. granulosa* which were *Bsal*-exposed; however, exhibited no to mild lethargy at the time of euthanasia were classified as "non-clinically diseased".

Experimental Infection

Bsal was isolated from a fire salamander (*Salamandra salamandra*) in the Netherlands and provided to my lab (isolate AMFP13/1, (Martel et al., 2013). Cultures were

maintained at the University of Tennessee Center for Wildlife Health laboratory and zoospores were enumerated as previously described by Carter et al. (2019).

Bsal-exposed individuals were exposed to a dose of $1x10^7$ zoospores per 10 mL by being individually placed into a 100-mL plastic tube containing a mixture of *Bsal* zoospores and sterile water for 24 hours in the environmental incubators. Control animals were placed into 100-mL plastic tubes containing 10-mL of sterile water under the same time and environmental conditions as the exposed individuals.

Sample Collection

Post-exposure, animals were skin swabbed once every 3 days throughout the study for an estimate of pathogen load. Additionally, skin swabs were collected for all animals at the time of necropsy. Immediately following euthanasia, tissues overlying the heart were carefully dissected using sterile instruments, and blood was collected directly from the heart using a sterile, heparinized capillary tube. Three to 5 microliters of blood were transferred onto a glass slide to make a blood smear using a gentle smear technique, five microliters of blood were pipetted into 995 microliters of filtered Natt-Herrick solution in a 1.5 milliliter sterile plastic conical tube, and the remaining blood sample was placed into a sterile 1.5 milliliter plastic conical tube. Blood samples in conical tubes were kept in a cooler on ice immediately after collection and transported to the University of Tennessee College of Veterinary Medicine (UTCVM) clinical pathology laboratory for processing. A sample of skin from the ventrum as well as a toe from each animal were collected and placed into separate 1.5 milliliter conical tubes. All skin swabs along with the skin and toe samples were placed immediately into a -80°C freezer. Carcasses were placed whole into 10% neutral buffered formalin.

Hematobiochemical Analyses

We processed all blood samples in the University of Tennessee College of Veterinary Medicine (UTCVM) clinical pathology lab. Packed cell volume (PCV) was assessed using whole blood samples. White blood cell differentials were performed on blood smears stained with Wright's Giemsa stain, and white blood cell/red blood cell/platelet counts were performed on the blood in the Natt-Herrick solution using a hemocytometer as outlined in Nardini et al. (2013). Cell counts were performed within 48 hours of blood being mixed with Natt-Herrick solution. Whole blood was spun in an Eppendorf 5424R centrifuge at 2170 G for 3 minutes to separate plasma. Plasma was analyzed using a Roche cobas c501 analyzer assessing the sodium (Na), potassium (K), chloride (Cl), bicarbonate (CO2), total protein, magnesium (Mg), calcium (Ca), phosphorous, alkaline phosphatase (ALP), creatinine kinase (CK), cholesterol, blood urea nitrogen (BUN), glucose, lactate dehydrogenase (LDH), as well as the hemolytic, lipemic, and icteric indices. Remaining plasma was stored in a 2°C refrigerator until it was used for agarose gel electrophoresis (<24 hours after collection).

Plasma Protein Electrophoresis

Plasma samples were analyzed in accordance with instructions provided by the Helena QuickGel system with the use of Split Beta gels (Helena Laboratories, Inc., Beaumont,

Texas 77707, USA). Samples were run in duplicate (average mean variance between duplicates = 0.006) and with a known human control sample. Gels were scanned and analyzed using software provided by Helena.

Histopathology

Carcasses were stored in 10% neutral buffered formalin for a minimum of 48 hours prior to processing. Transverse sections through the entire body were taken and placed into tissue cassettes following the protocol listed in the Appendix (Table 1.1). Cassettes containing tissues were decalcified for 24 hours using a formic acid solution. Tissues were then routinely processed and stained with hematoxylin and eosin (H&E). Histopathology consisted of examining all tissues for any abnormalities as well as performing lesion counts and grading on all sections. Lesion grading consisted of scoring each lesion on a scale of 1–5 based on its depth as well as length. Grade 1 lesions were limited to *Bsal* organisms within the stratum corneum, grade 2 lesions included *Bsal* organisms and associated cellular damage which extended into the mid-epidermis, grade 3 lesions included *Bsal* organisms and associated cellular damage which extended full thickness through the epidermis, grade 4 lesions included coalescing/large lesions in which *Bsal* organisms and associated cellular damage extended full thickness through the epidermis and were <1 millimeter in length, grade 5 lesions included coalescing/large lesions in which *Bsal* organisms and associated cellular damage extended full thickness through the epidermis and were ≥ 1 millimeter in length (Table 1.2 & Figure 1.4). In order to standardize measures of lesion counts to counts per unit area of cross section, the perimeter of each section was determined using Excelis Accu-Scope software.

Presence or absence of inflammation associated with skin lesions was documented for each exposed individual. Additionally, inflammation within each *Bsal*-associated lesion was estimated as mild (<30% of the lesion infiltrated by inflammatory cells), moderate (30–60% of the lesion infiltrated by inflammatory cells), or severe (>60% of the lesion infiltrated with inflammatory cells), and an overall severity grade was assigned to each individual based on which grade of inflammation occurred most frequently. Also, areas of inflammation associated with *Bsal* but not bacteria were counted for each individual.

Quantitative Polymerase Chain Reaction (qPCR)

To detect *Bsal* and estimate loads, genomic DNA was extracted from each skin swab using the QIAamp 96 DNA QIAcube HT kit (Qiagen, Hilden, Germany) and qPCR was performed similar to Blooi et al (2015) using the Applied Biosystems Quantstudio 6 Flex qPCR instrument (Thermo Fisher Scientific Inc). All samples were run in duplicate and declared positive if both replicates reached cycle threshold prior to 50 amplification cycles (Carter et al., 2019).

Statistical Analysis

Biochemical, Hematological, and Plasma Protein Electrophoresis Measurands

To determine how clinical disease status affected biochemical values, I performed a principal component analysis (PCA) using biochemical values followed by a multivariate

analysis of variance (MANOVA) to determine which values were significantly different between clinically diseased and non-clinically diseased *T. granulosa*. Biochemical values included in the analysis were alkaline phosphatase (ALP), blood urea nitrogen (BUN), phosphorous, calcium, glucose, protein, cholesterol, sodium (Na), chloride (Cl), bicarbonate (CO2), anion gap, magnesium, creatinine kinase (CK), lactate dehydrogenase (LDH), and potassium (K). Hemolytic index (H) was also measured for each sample. Increased hemolysis in a sample can lead to artifactual elevations in CK, LDH, and K. Therefore, I independently regressed CK on H, LDH on H, and K on H and extracted the residuals of these regressions. These residuals account for the variation in CK, LDH, and K not described by changes in hemolytic index (H). I used these new variables in place of original CK, LDH, and K values in my analyses. Shapiro-Wilk normality tests were performed on all variables to assess for normality, and variables which failed this test were log transformed. If the variable still failed the normality test once log transformed, then the original value was used.

I scaled my data (e.g., zero mean and standard deviation of one) and used K means clustering with the elbow method to determine the optimal number of clusters to use in order to group individuals based on similarity in their blood chemistry values (Yuan & Yang, 2019). A PCA performed on these clusters highlighted that the majority of clinically diseased animals clustered together, and the majority of non-clinically diseased animals clustered together (Figure 1.5). I then ran a MANOVA to test which blood chemistry values significantly differed between clinically diseased and non-clinically diseased animals. Wilks' statistic was used to interpret the overall significance of the MANOVA due to its robustness towards lack of normality and homogeneity of variance (Ates et al., 2019). I examined significant differences between each blood chemistry value for diseased and non-diseased individuals. To account for type I error rate, I used false discovery rate to correct p-values obtained from the MANOVA for specific chemistry variables (Benjamini & Hochberg, 1995). I used a family-wise type I error rate of $\alpha = 0.05$ for all analyses. Arithmetic means for each value were compared between clusters. All analyses were run in R (version 4.1.1).

To determine how *Bsal* qPCR load at the time of necropsy affected changes in biochemical values, a permutational multivariate analysis of variance (PERMANOVA) was performed using the vegan package (Oksanen et al., 2020). Biochemical values were used as the dependent variable and *Bsal* load at the time of necropsy as the independent variable. The log of *Bsal* load +1 was used as some of the load values were 0. I followed this analysis with correlation tests between each individual variable and *Bsal* load using Pearson's product moment correlation and corrected with false discovery rate.

A similar set of analyses as described above was performed for hematological values (PCA presented in Figure 1.6) and for plasma protein electrophoresis values (PCA presented in Figure 1.7). Hematological values included in the analysis were absolute counts of segmented neutrophils (ACSegNeut), band neutrophils (ACBands), lymphocytes (ACLymphs), monocytes (ACMonos), eosinophils (ACEos), and basophils (ACBasos). For variables which included zeros, the log of the variable +1 was used. Plasma protein electrophoresis results included 8 fractions (Avr1–8). Increased hemolysis in a sample can lead to artifactual alterations in various plasma protein fractions in other

species (Cray, 2021). Therefore, I independently regressed each fraction on the hemolytic index of the sample (H). For each regression in which H was determined to significantly explain variation in the plasma protein fraction (fractions 3, 5, and 8), I extracted the residuals of these regressions. These residuals account for the variation in each of these fractions not described by changes in hemolytic index (H). I used these new variables in place of the original protein fractions in my analyses. A Shapiro-Wilk normality test was performed on all variables to assess for normality, and variables which failed this test were log transformed.

Food Consumption

To determine how treatment (control versus *Bsal*-exposed) affected food consumption in *T. granulosa*, I fit a binomial generalized linear mixed effects model where the response variable was the combined vector of food consumed and food not consumed, and the predictor variable was the interaction term of time and treatment with the random effect of individual ID. I fit the model using the lme4 package in R (version 4.1.1). I fit two candidate models to the data including a model similar to the one described above; however, time and treatment were included as main effects instead of as an interaction term. The two models were compared using Akaike information criteria (AIC), where lower AIC indicates better predictive performance. Only exposed, clinically diseased animals and controls were included in this analysis due to variations in survival time of exposed, non-clinically diseased individuals.

Lesion Count

To determine how *Bsal* qPCR load at the time of necropsy affected lesion count, I fit a negative binomial generalized linear model where the response variable was lesion count per cross section perimeter and the predictors were log(*Bsal* load), section number, and the random effect of individual ID. Section number included sections 1–10, corresponding to different regions of the animal's body (Table 1.1). I used a negative binomial distribution to describe lesion counts as initial analyses showed that lesion counts were over-dispersed relative to a Poisson distribution. In addition, lesion counts were obtained from histological cross sections with different perimeters, where a larger perimeter could lead to a higher number of counted lesions than a smaller perimeter even if the density of lesions per unit perimeter were the same. To account for this, I included an offset term of log(total perimeter) in the negative binomial regression (Faraway, 2016), which allowed me to model the mean density of lesions per cross section rather than the total abundance. I fit the lesion count model using the brms package in R (Burkner, 2017) (version 4.1.1), with a weakly regularizing prior on the effect sizes for the predictor variables log(*Bsal* load) and section number. When fitting the models, I ensured all convergence by visually examining chains and checked that R_hat (the potential scale reduction factor) < 1.01 and effective sample size > 400 for all parameters (Vehtari et al., 2020). I fit five candidate models to the data and selected the best predictive model using Pareto smoothed importance sampling leave-one-out information (PSIS-LOO) (Vehtari et al., 2016). Models with lower PSIS-LOO indicate better predictive performance. I compared models including the following combinations of

predictors to my final model described above: 1) log(*Bsal* load), 2) log(*Bsal* load) and section number, 3) log(*Bsal* load) and the random effect of individual ID, and 4) the interaction term of log(*Bsal* load) and section number along with the random effect of individual ID.

3. Results

Biochemical Values

There was a significant difference in biochemical values between the two clusters corresponding to clinically diseased and non-diseased individuals (MANOVA: Wilks' statistic = 0.13, p= 2.27e-06). Na (F= 53.76, p = 1.21e-08), Cl (F= 52.99, p = 1.42e-08), CO2 (F= 17.99, p= 0.0001), and cholesterol (F= 9.79, p=0.0035) were significantly decreased on average in clinically diseased animals compared to non-clinically diseased animals (Figure 1.8) and K (F= 11.01, p= 0.0021), ALP (F= 24.65, p= 1.673e-05), anion gap (F= 29.97, p=3.5e-06), total protein (F= 10.41, p= 0.00027), and LDH (F= 4.91, p=0.0332) were significantly increased (Figure 1.8).

There was a significant relationship between biochemical values and *Bsal* load (PERMANOVA: F= 14.97, p = 0.01). The variables Na, Cl, K, and anion gap were significantly correlated with *Bsal* load at the time of necropsy (Na: r=-0.51 95%CI: -0.71– -0.22 Cl: r=-0.53, 95%CI: -0.73– -0.26; K: r=0.45, 95%CI: 0.15–0.67; Anion gap: r=0.45, 95%CI: 0.15–0.67). As necropsy *Bsal* load increased, K and anion gap increased, and Na and Cl decreased (Figure 1.9).

Hematological Values

There was a significant difference in hematological values between diseased and non-diseased clusters (MANOVA: Wilks' statistic: 0.22, p = 2.26e-10). Segmented neutrophils (F = 27.84, p = 4.59e-06), band neutrophils (F= 54.07, p = 5.26e-09), monocytes (F= 12.64, p = 0.0009), and eosinophils (F= 17.98, p = 0.0001) were significantly increased in the majority of clinically diseased animals compared to non-clinically diseased animals (Figure 1.10).

Mild toxic change was identified in neutrophils of 3 exposed, clinically diseased along with 1 control *T. granulosa*. Döhle bodies (1+) were identified in 1 exposed, clinically diseased and 1 control *T. granulosa*. While trends in the means of PCV showed differences between controls, exposed/non-clinically diseased and exposed/clinically diseased *T. granulosa* (Figure 1.11), only two clinically diseased individuals had PCV measurements and thus a formal statistical analysis was not performed. Small sample size for PCV analysis was due to limitations with blood volume collected.

Plasma Protein Electrophoresis

There was a significant difference in plasma protein electrophoresis values between the diseased and non-diseased clusters (MANOVA: Wilks' statistic: 0.17, p = 8.884e-12). Protein fractions two (F= 20.74, p=1.1e-04) and three (F= 40.67, p = 7.51e-07) were on average significantly increased in clinically diseased animals compared to non-clinically diseased animals, and fractions one (F=14.24, p=6.37e-04), five (F= 34.53, p= 2.05e-06),

six (F= 18.32, p= 1.99e-04), seven (F= 15.45, p= 4.75e-04), and eight (F= 7.54, p= 9.96e-03) were decreased (Figure 1.12). Figure 1.13 shows an example electrophoretogram from a clinically diseased and a control *T. granulosa*.

Food Consumption

I identified a strong interaction between time and *Bsal* exposure on food consumption in control versus clinically diseased *T. granulosa* (E: -0.92, z-value = -2.59, p = 0.0094). The food consumption of control individuals remained consistent over time, while food consumption of *Bsal*-exposed individuals decreased (Figure 1.14). This was supported by our model comparison – the AIC of the model including time*treatment was 5.44 units lower than the model including time + treatment.

Anatomic Pathology

Lesion Counts

A significant positive relationship was identified between lesion count and *Bsal* load at necropsy (Figure 1.15), with the best model (i.e., the model with the lowest PSIS-LOO) including $\log_{10}$(*Bsal* load), section number, and the random effect of individual ID as predictors. Other candidate models included the following predictors, with the difference in PSIS-LOO between the best model and each candidate model listed: (1) $\log_{10}$(*Bsal* load)*section number + random effect of individual ID = 1.1, (2) $\log_{10}$(*Bsal* load) + random effect of individual ID = 18.5, (3) log(*Bsal* load) = 349.4, (4) $\log_{10}$(*Bsal* load) and section number = 354.2. My model predicted that a one unit increase in $\log_{10}$(*Bsal* load) led to a 2.84X increase in lesion count (95% CI: 2.01–4.45).

Inflammation Associated with Skin Lesions

Inflammation was associated with *Bsal* skin lesions in 46% of *Bsal*-exposed individuals. Inflammation was frequently associated with *Bsal* lesions which also had secondary bacterial invasion. All inflammatory lesions associated with *Bsal* infection consisted of mononuclear as well as granulocytic inflammatory cells.

Dermal Glands and Internal Organs

Varying degrees of dermal gland invasion by *Bsal* organisms (from 1–13 glands total) were identified histologically in 75% of clinically diseased *T. granulosa*. Dermal gland invasion was only documented in one individual that was not classified as clinically diseased; however, did exhibit mild skin sloughing. This individual had *Bsal* organisms present in 4 dermal glands. All areas of dermal gland invasion had *Bsal* organisms and associated cellular damage in the overlying dermis and epidermis which was variably eroded to ulcerated. No lesions related to *Bsal* chytridiomycosis were identified in internal organs.

4. Discussion

The objective of this study was to elucidate the pathogenesis of *Bsal* chytridiomycosis through comparison of clinical presentation as well as clinical and anatomic pathology changes in diseased versus control individuals. I identified that electrolyte imbalances

and dehydration likely play a key role in disease. I also found evidence of a systemic inflammatory response which may be in response to the *Bsal* organism itself, or due to secondary bacterial infection of *Bsal*-induced skin lesions.

Clinically diseased *T. granulosa* had significantly decreased blood Na and Cl levels and increased blood K levels compared to control and aclinically infected *T. granulosa*. Changes in Na and Cl are similar to those reported in *Bd* chytridiomycosis (Marcum et al., 2010; Voyles et al., 2007; Voyles et al., 2009). However, in contrast to my findings in *Bsal* chytridiomycosis, previous studies have reported decreased K levels in clinically diseased individuals with *Bd* chytridiomycosis compared to control and aclinically infected individuals (Marcum et al., 2010; Voyles et al., 2007; Voyles et al., 2009).

The difference in distribution of lesions within the epidermal layers between *Bd* and *Bsal*, along with primarily immature sporangia infecting this critical layer in *Bd* infections, may contribute to the differences noted in potassium alterations between the two pathogens. *Bd* thalli infect epidermal cells of the stratum corneum and stratum granulosum, with mature sporangia infecting cells of the stratum corneum and immature sporangia infecting the stratum granulosum (Berger et al., 2005). The stratum granulosum is the layer of the epidermis most prominently involved in the active transport of electrolytes and regulates electrolyte balance and osmoregulation in amphibians (Campbell et al., 2012; Larsen, 1991). In contrast, *Bsal* thalli including mature forms frequently extend into deeper layers of the epidermis and in severe lesions, the epithelial layer is absent due to ulceration (Pessier, 2018). Therefore, the extent of damage to this layer of the epithelium is likely more extensive in *Bsal*-infected individuals, which may lead to the differences in K values seen between these two pathogens.

Additionally, studies investigating electrolyte imbalances with *Bd* chytridiomycosis have utilized anurans as the model species as the majority of *Bd* chytridiomycosis declines have occurred in these species (Scheele et al., 2019). However, my study used a urodelan as the model, since urodelans have been shown to be more susceptible to *Bsal* infection (Martel et al., 2014). It has been shown that ion transport characteristics, particularly involving potassium, can vary between species and also with environmental conditions in amphibians (Nagel & Hirschmann, 1980). Additionally, the vast majority of what we know about electrolyte transport within amphibian epidermal cells is based on studies in anurans. Therefore, it is possible that there are innate differences in epidermal electrolyte transport between the species used in these studies.

Another important finding in clinically diseased *T. granulosa* was increased total protein in biochemical analyses along with increased fractions 2 and 3 and secondarily decreased fractions 1, 5, 6, 7, and 8 in plasma protein electrophoretograms. Fraction 2 is believed to represent albumin based on an extrapolation of what is known in mammalian and more well-studied non-mammalian species. Increase in this fraction is most commonly associated with dehydration, especially in conjunction with increased total protein (Zaias & Cray, 2002). Previous studies with *Bd* have not shown changes in total protein levels in clinically diseased individuals (Marcum et al., 2010; Voyles et al., 2007; Voyles et al., 2009). However, some studies have shown evidence that dehydration may play a role in *Bd* chytridiomycosis pathogenesis. Young, et al. (2012) found that fluid

therapy is useful to incorporate in successful treatment regimens for *Bd* chytridiomycosis. Additionally, another study provided evidence that clinically diseased *Litoria ranifomis* experienced slower rates of rehydration than aclinically infected individuals after *Bd* exposure, which may provide evidence that aquaporin pumps within the skin are impaired in diseased individuals (Carver et al., 2010).

Plasma protein fraction 3 in *T. granulosa* corresponds with the alpha-1 globulin fraction in other species. An increase in alpha-1 globulins is associated with either acute inflammation or parasitism in avian species (Zaias & Cray, 2002). As no parasites were identified in *T. granulosa* in this study, acute inflammation is the most likely cause of this trend seen in clinically diseased individuals. This premise is also supported by the complete blood count results. Clinically diseased *T. granulosa* had increased segmented neutrophils, band neutrophils, monocytes, and eosinophils compared to control and aclinically diseased *T. granulosa*. These increases likely represent an acute inflammatory response; however, it is uncertain whether this response is due to the *Bsal* infection itself or to a secondary bacterial infection (Bletz et al., 2018).

One particularly interesting feature of the alterations identified in complete blood counts of clinically diseased *T. granulosa* is that they exhibited a left shift with increased numbers of band (immature) neutrophils in circulation. The presence of a left shift is most commonly associated with inflammation, and occurs when inflammatory cytokines stimulate neutrophil production and release of mature and immature neutrophils from hematopoietic tissues (Latimer & Duncan, 2011; Stacy et al., 2022). The increase in band neutrophils in addition to increase in segmented (mature) neutrophils and other complete blood cell count findings further support this as an inflammatory response.

Another indication of an inflammatory response was that mild toxic change was identified in neutrophils of three clinically diseased *T. granulosa*, and a small number of Döhle bodies were identified in another clinically diseased *T. granulosa*. Toxic change in non-mammalian vertebrate species is similar to that in mammalian species and is caused by rapid release of neutrophils from hematopoeitic tissues into circulation which shortens their maturation time (Stacy et al., 2022). Toxic change and a left shift often occur simultaneously as signs of an inflammatory response (Stacy et al., 2022). Döhle bodies can also be an indication of accelerated maturation. All the clinically diseased *T. granulosa* who had a mild toxic change or the presence of Döhle bodies also had a left shift. Mild toxic change and a small number of Döhle bodies were also identified in two separate control individuals. Therefore, the significance of these findings is uncertain. Phagocytosed material or artifactual granulation due to suboptimal sample handling, etc. can be mistaken for Döhle bodies. Additionally, suboptimal sample handling or staining can lead to other characteristics of toxic change. Therefore, further investigation of these changes is warranted to determine their significance.

To the author's knowledge, this is the first study investigating changes in complete blood count values associated with *Bsal* chytridiomycosis. Studies with *Bd* chytridiomycosis have shown mixed results regarding alterations in circulating white blood cells associated with infection. Davis, et al. (2010) and Peterson, et al. (2013) showed neutrophilia and eosinopenia in *Bd*-infected animals. Peterson, et al. (2013) also showed lymphocytopenia. Woodhams, et al. (2007) showed neutropenia and eosinopenia

with basophilia in response to *Bd* infection. Overall, none of these previous studies with *Bd* have identified a similar inflammatory pattern to that seen in this study.

Previous studies of *Bd* and *Bsal* chytridiomycosis have shown no to minimal inflammation associated with skin lesions histologically. Overall, inflammation is uncommon, and when seen, is often associated with *Bd* tolerant species or with secondary bacterial or fungal infections (Pessier, 2018). In this study, inflammation was identified histologically within *Bsal*-associated skin lesions in 46% of *Bsal*-exposed *T. granulosa*. The vast majority of inflamed lesions also contained bacteria. Inflammation was frequently more severe in individuals that developed lesions, shed/sloughed their skin and re-developed lesions without developing any evidence of lethargy or hemorrhage, whereas inflammation was mild to absent in clinically diseased individuals. It is uncertain whether this inflammatory response was directed at the *Bsal* organisms, or to the intralesional bacteria identified. Hence, it remains unclear whether bacteremia could contribute to *Bsal* pathogenesis (Bletz et al., 2018).

The majority of clinically diseased animals either died or were euthanized within 13 days post-exposure, and these individuals all either died or were euthanized within 24 hours of the onset of clinical disease. Therefore, it is possible that in these individuals, the immune system did not have time to appropriately respond to infection. Previous studies have provided evidence that *Bd* causes dysregulation of the local immune response within the skin, and that *Bsal* also possesses similar properties (Rollins-Smith, 2020; Rollins-Smith et al., 2022). It is possible that as the local immune response was inadequate to fight off the infection, the systemic immune response was initiated; however, the immune cells did not have time to reach their destination prior to mortality of the individual. This hypothesis is supported by the increase in circulating leukocytes along with a left shift identified in clinically diseased animals. It is also possible, however, that this immune response was secondary to bacterial infection.

Other alterations seen in biochemical values which are of interest include an increase in anion gap, LDH, and ALP, along with a decrease in bicarbonate and cholesterol in clinically diseased *T. granulosa*. Changes in anion gap and bicarbonate cannot be fully evaluated without the incorporation of blood gas analysis which was not performed in this study. This is because blood gas analysis evaluates the respiratory component of the acid-base status as well as the pH of the animal. Therefore, I can only make speculations based on the information I have.

Potential reasons for increased anion gap in clinically diseased *T. granulosa* include increased albumin due to dehydration or increased accumulation of lactic acid due to cellular damage (e.g., skin damage). Additionally, if respiration is compromised due to epidermal damage, increased carbon dioxide within the body could contribute to a respiratory acidosis (Latimer & Duncan, 2011). Reasons for decreased bicarbonate could include either an increase in carbon dioxide secondary to respiratory compromise, or metabolic acidosis (Latimer & Duncan, 2011). These results suggest that cutaneous respiration could be compromised in salamanders with *Bsal* chytridiomycosis, which may have minimal effects on species with lungs (as in our study) but could be a substantial contributor to pathogenesis in lungless species. Metabolic acidosis could be due to an increase in acids such as lactic acid or a loss of bicarbonate through the gastrointestinal

tract or kidneys. As no abnormalities were identified in the gastrointestinal or renal tract, this is unlikely in this case. Overall, future studies should incorporate blood gas analysis into diagnostic investigations of *Bsal* chytridiomycosis to further investigate the significance of these findings.

Lactate dehydrogenase can be increased due to tissue damage (e.g. skin damage) (Latimer & Duncan, 2011). It can also be increased in cases of liver or muscle injury and in some cases of neoplasia (Latimer & Duncan, 2011). No evidence of liver or muscle injury was identified; however, it is possible that hypoperfusion to these organs secondary to dehydration was occurring and leading to damage which was not yet visible histologically. Additionally, alkaline phosphatase can be increased in liver disease, particularly cholestasis (Fernandez & Kidney, 2007). It can also be increased in association with increases in corticosteroids due to stress in some species such as dogs (Fernandez & Kidney, 2007). Liver disease which was not evident histologically or increased corticosteroids secondary to the stress of being diseased could have contributed to this increase in clinically diseased *T. granulosa*. Measurement of corticosteroid levels would be useful in future investigations to help understand the contribution that stress could have on biochemical as well as complete blood count values in *Bsal*-infected individuals.

Clinically diseased *T. granulosa* had decreased cholesterol compared to control and aclinically infected *T. granulosa*. This is suspected to be secondary to decreased food intake, as exposed, clinically diseased individuals consumed less food over time than control individuals in this study. This is an important finding, because malnutrition due to decreased food consumption in *Bsal*-infected individuals could contribute to morbidity and mortality (Carter et al., 2019).

The strong, positive relationship between *Bsal* qPCR load at necropsy and histologic lesion count shows that qPCR results are a good indicator of disease severity in *T. granulosa*. This is important as qPCR can be used as an antemortem diagnostic test which is non-invasive since samples are easily collected through swabbing the skin. It has been shown previously that enumeration of grossly visible skin lesions associated with *Bsal* infection is only weakly, positively correlated with *Bsal* qPCR load at the time of necropsy in *Taricha granulosa* as well as three other salamander species including *Notophthalmus viridescens*, *Notophthalmus meridionalis*, and *Notophthalmus perstriatus* (Wilber et al., 2021). As tolerance and resistance to infection can vary among species, it is important for additional studies to utilize histologic lesion counts along with qPCR results in *Bsal* studies across multiple species (Thomas et al., 2018).

Another important result related to *Bsal* qPCR load at necropsy is that as load increased, Na and Cl decreased, and K and anion gap increased. This further confirms the proposed pathogenesis of *Bsal* chytridiomycosis involving increased pathogen load leading to increased lesion count. Increased epidermal damage due to increased lesion count, then leads to alterations in blood chemistry and ultimately clinical disease.

As has been reported in previous chytridiomycosis studies, no internal lesions related to *Bsal* infection were identified (Pessier, 2008; Van Rooij et al., 2015). This further supports the hypothesis that skin lesions are the primary cause of morbidity and mortality in *Bsal* chytridiomycosis. Histologic lesions associated with electrolyte

imbalances and dehydration are often not present in internal organs especially in acute cases of morbidity and mortality as was seen in this study. Indeed, the lack of internal lesions provides important insight into *Bsal* pathogenesis.

Another potential contributor to *Bsal* disease pathogenesis is damage to the dermal glands. Seventy-five percent of clinically diseased *T. granulosa* had some degree of dermal gland invasion, whereas among aclinically diseased *T. granulosa*, only one individual had evidence of dermal gland invasion. Dermal glands serve important functions such as maintaining moisture level of the skin, secreting antimicrobial peptides to inhibit infections, and producing toxins to deter predators (Varga et al., 2018). Previous studies have shown infection of dermal glands can occur in eastern newts (*Notophthalmus viridescens*) with *Bsal* chytridiomycosis, and it is suspected that this may play a role in disease progression (Ossiboff et al., 2019). Investigation of compromise to dermal gland function with *Bsal* infection is warranted in future studies to better understand the role this may play in disease pathogenesis.

Overall, this study provides insight into the pathogenesis of *Bsal* chytridiomycosis through utilization of multiple diagnostic techniques which have not yet been used together in investigation of this disease. Based on my findings, there is evidence that *Bsal*-induced skin lesions lead to electrolyte imbalance and dehydration. It is possible that secondary bacterial infections and damage to dermal glands play a role in pathogenesis as well. Similar investigations involving additional species with various levels of *Bsal* infection tolerance are warranted to better understand the host response to disease. Additionally, studies utilizing additional diagnostic techniques, such as blood gas analysis and corticosteroid measurement, would be useful to determine what role respiratory compromise and stress response play in disease progression.

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APPENDICES

Appendix A: Tables

Table 1.1 Anatomic location associated with each of ten standardized sections examined histologically for lesion counts and grading in *Batrachochytrium salamandrivorans* infected *Taricha granulosa*.

Section Number	Anatomic Location
1	Cranial to eye
2	Caudal to eye
3	Cranial to thoracic limbs
4	Caudal to thoracic limbs
5	Left forelimb
6	Right hindlimb
7	Mid-body
8	Cranial to hindlimbs
9	Caudal to hindlimbs
10	Caudal 3 cm of the tail

Table 1.2 Description of histologic lesion grading system (1–5) used in *Batrachochytrium salamandrivorans* infected *Taricha granulosa*.

Lesion Grade	Description
1	Lesion/Organisms extending into the stratum corneum only
2	Lesion/organisms extending to the level of the mid-epidermis
3	Lesion/organisms extending through the full thickness of the epidermis
4	Coalescing/large lesion which extends full thickness of the epidermis and is <1 millimeter in length
5	Coalescing/large lesion which extends full thickness of the epidermis and is ≥ 1 millimeter in length

Appendix B: Figures

Figure 1.1 *Batrachochytrium salamandrivorans* exposed, clinically diseased *Taricha granulosa* with severe, multifocal skin sloughing especially prominent on the chin, neck, legs, feet, and tail. (TW49)

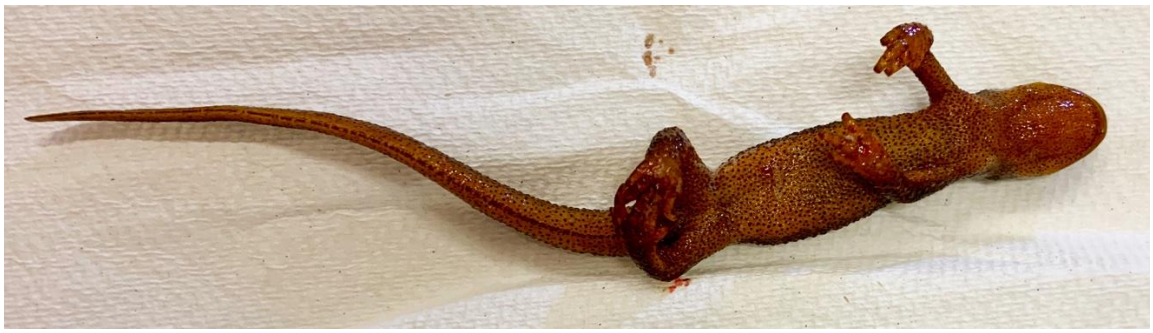

Figure 1.2 *Batrachochytrium salamandrivorans* exposed, clinically diseased *Taricha granulosa* with multifocal hemorrhage on the chin, legs, and feet. (TW25)

Figure 1.3 *Taricha granulosa* with multiple, characteristic erosive to ulcerative *Batrachochytrium salamandrivorans* induced skin lesions. **(**TW14)

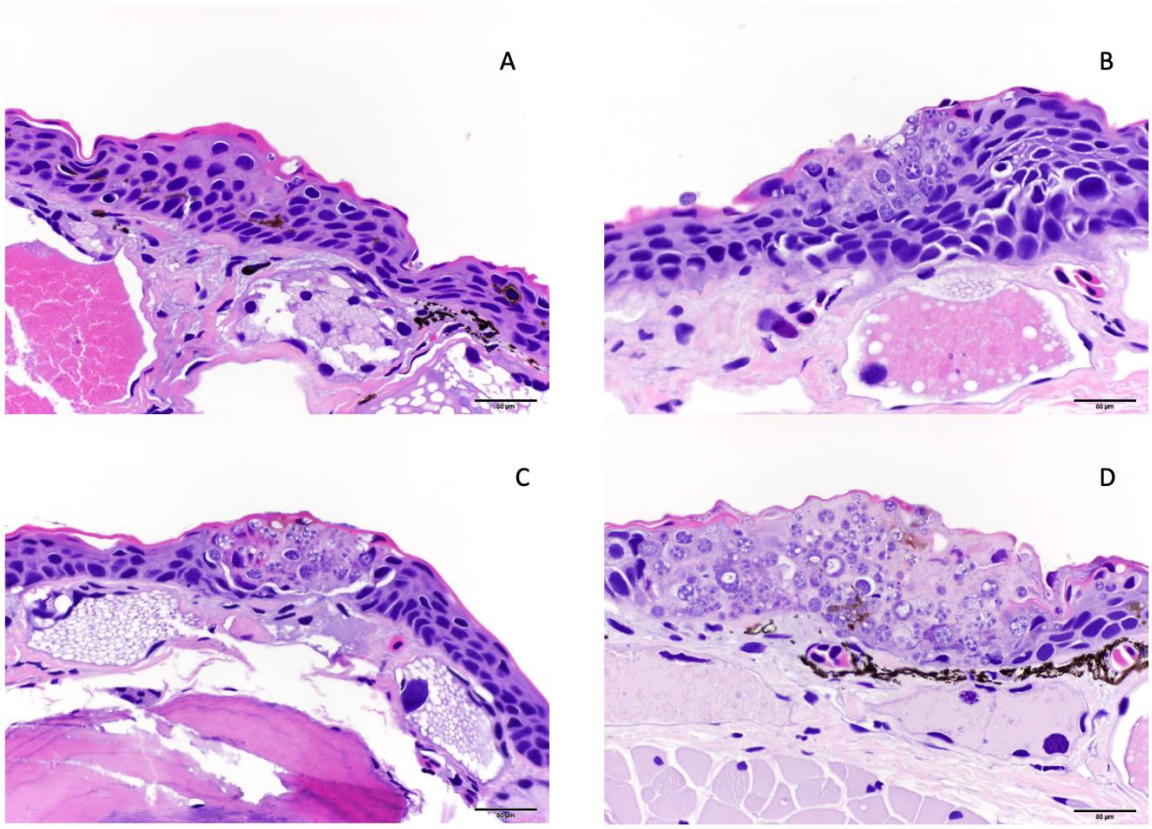

Figure 1.4 Examples of histologic lesion grading scheme used for *Taricha granulosa* infected with *Batrachochytrium salamandrivorans* (*Bsal*) including Grade 1 (A), Grade 2 (B), Grade 3 (C), and Grade 4 (D). Grade 1 lesions were limited to *Bsal* organisms within the stratum corneum, grade 2 lesions included *Bsal* organisms and associated cellular damage which extended into the mid-epidermis, grade 3 lesions included *Bsal* organisms and associated cellular damage which extended full thickness through the epidermis, grade 4 lesions included coalescing/large lesions in which *Bsal* organisms and associated cellular damage extended full thickness through the epidermis and were <1 millimeter in length.

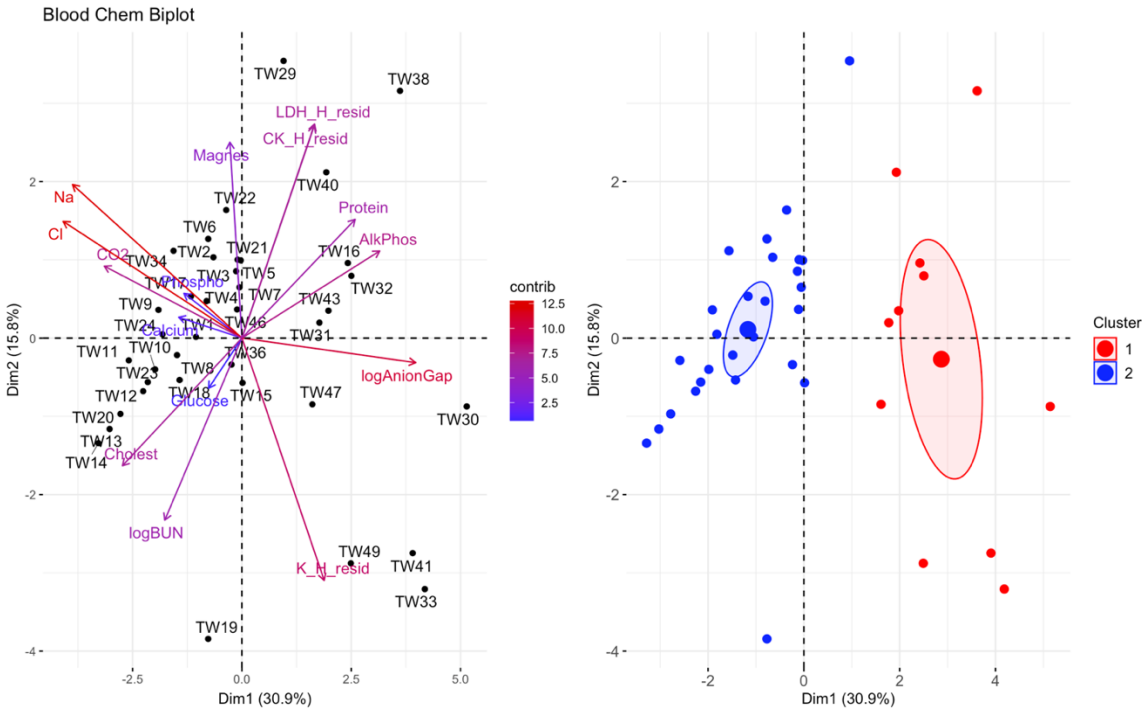

Figure 1.5 Principal component analysis (PCA) for blood chemistry data. In the biplot on the left, individuals in the same space as the arrowhead had higher values of the blood parameter, and individuals in the opposite direction had lower levels of the blood parameter. The length and color of each arrow represent the strength of the relationship. The plot on the right is showing how the individuals clustered together using K means clustering. The majority of *Taricha granulosa* in cluster 1 (red) were *Batrachochytrium salamandrivorans* (*Bsal*) infected, clinically diseased individuals, and the majority of *T. granulosa* in cluster 2 (blue) were controls or *Bsal*-infected, non-clinically diseased individuals. Sodium = Na; Chloride = Cl; Cholesterol = cholest; Blood urea nitrogen = logBUN; Phosphorous = Phospho; Alkaline phosphatase = AlkPhos; Magnesium = Magnes; the residuals of potassium (K) regressed on hemolytic index (H) = (K_H_resid); the residuals of creatinine (CK) regressed on H = (K_H_resid); the residuals of lactate dehydrogenase (LDH) regressed on H = (K_H_resid).

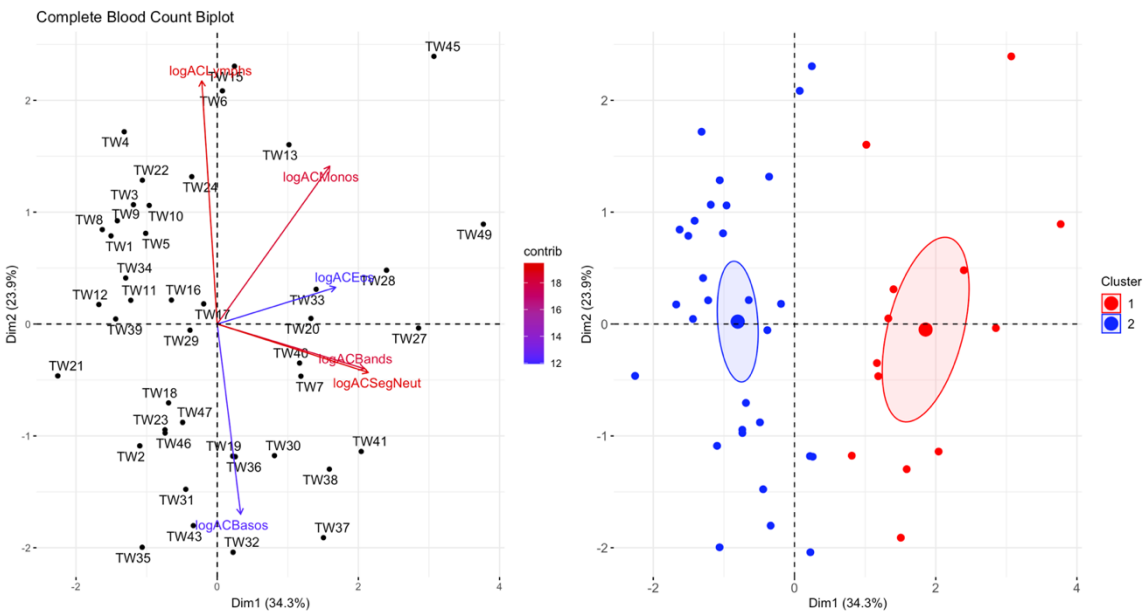

Figure 1.6 Principal component analysis (PCA) for the complete blood count (CBC) data. In the plot on the left, individuals in the same space as the arrowhead had higher values of the blood parameter, and individuals in the opposite direction had lower levels of the blood parameter. The length and color of each arrow represent the strength of the relationship. The plot on the right is showing how the individuals clustered together using K means clustering. The majority of *Taricha granulosa* in cluster 1 (red) were *Batrachochytrium salamandrivorans* (*Bsal*) infected, clinically diseased individuals, and the majority of *T. granulosa* in cluster 2 (blue) were controls or *Bsal*-infected, non-clinically diseased individuals. Absolute count of lymphocytes = logACLymphs; absolute count of monocytes = logACMonos; absolute count of eosinophils = logACEos; absolute count of band neutrophils = logACBands; absolute count of segmented neutrophils = logACSegNeut; absolute count of basophils = logACBasos.

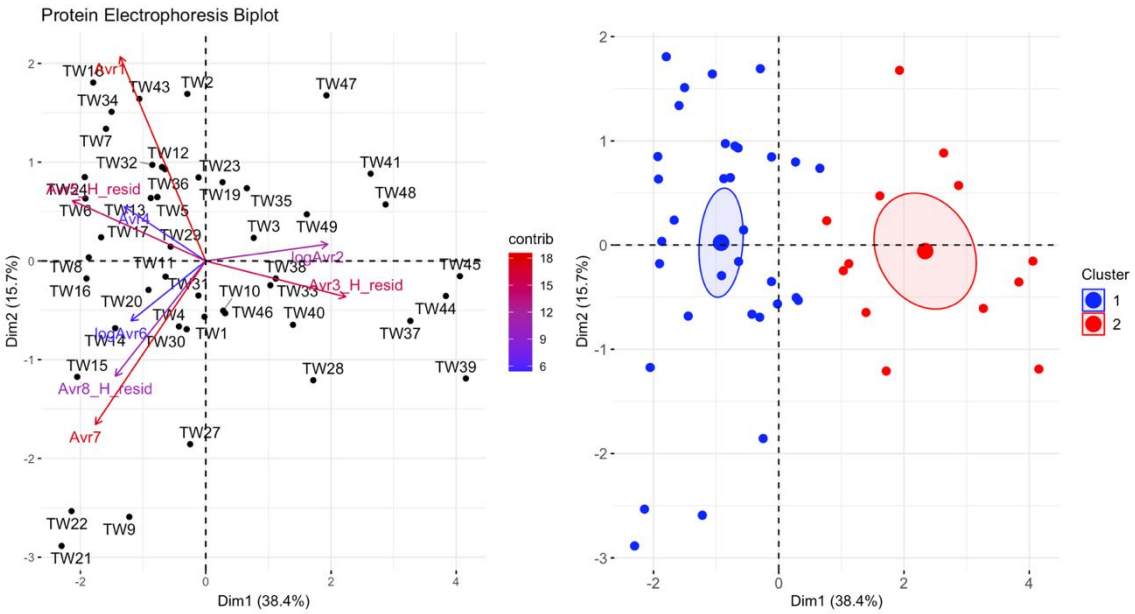

Figure 1.7 Principal component analysis (PCA) for the plasma protein electrophoresis data. In the plot on the left, individuals in the same space as the arrowhead had higher values of the blood parameter, and individuals in the opposite direction had lower levels of the blood parameter. The length and color of each arrow represent the strength of the relationship. The plot on the right is showing how the individuals clustered together using K means clustering. The majority of *Taricha granulosa* in cluster 1 (blue) were controls or *Batrachochytrium salamandrivorans (Bsal)* infected, non-clinically diseased individuals, and the majority of *T. granulosa* in cluster 2 (red) were *Bsal*-infected, clinically diseased individuals.

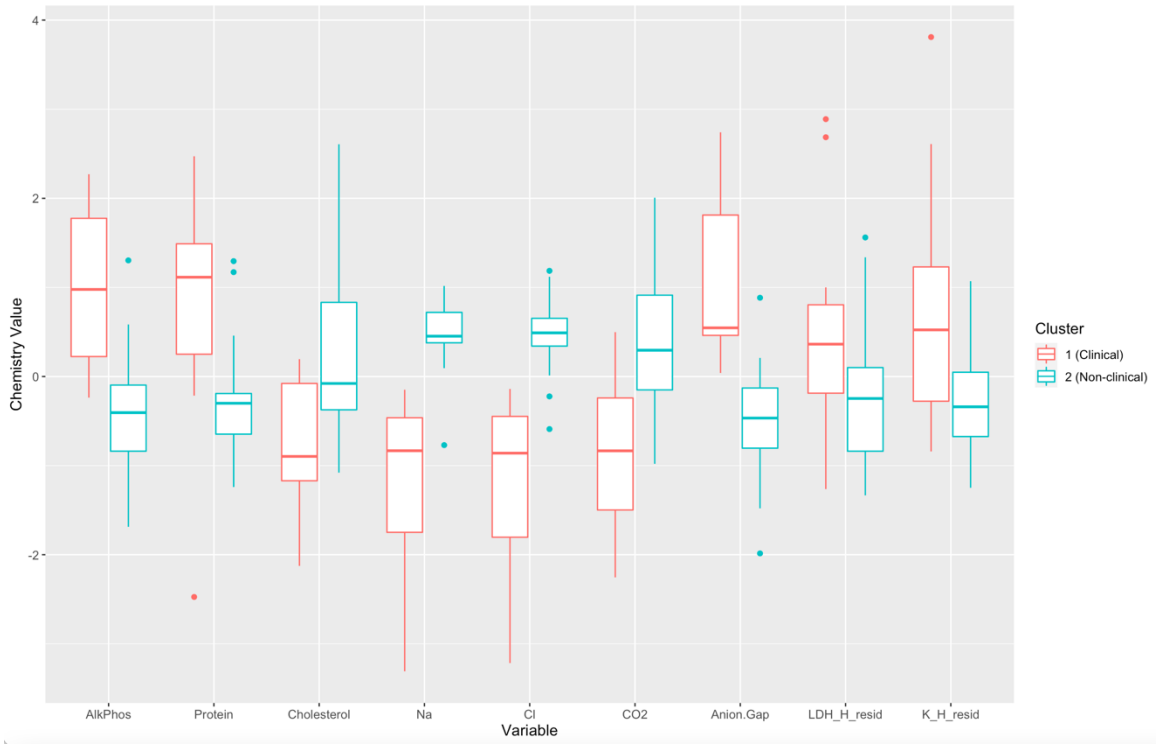

Figure 1.8 Boxplots comparing each blood chemistry variable for cluster 1 (*Batrachochytrium salamandrivorans* (*Bsal*) infected, clinically diseased *Taricha granulosa*) in red and cluster 2 (control and *Bsal*-infected, non-clinically diseased *T. granulosa*) in blue. Each box represents the interquartile range (IQR), the horizontal line within each box represents the median, vertical lines represent 1.5 * IQR, and dots represent outliers. Alkaline phosphatase = AlkPhos; sodium = Na; chloride = Cl; bicarbonate = CO2; the residuals of lactate dehydrogenase (LDH) regressed on hemolytic index (H) = LDH_H_resid; the residuals of potassium (K) regressed on H = K_H_resid.

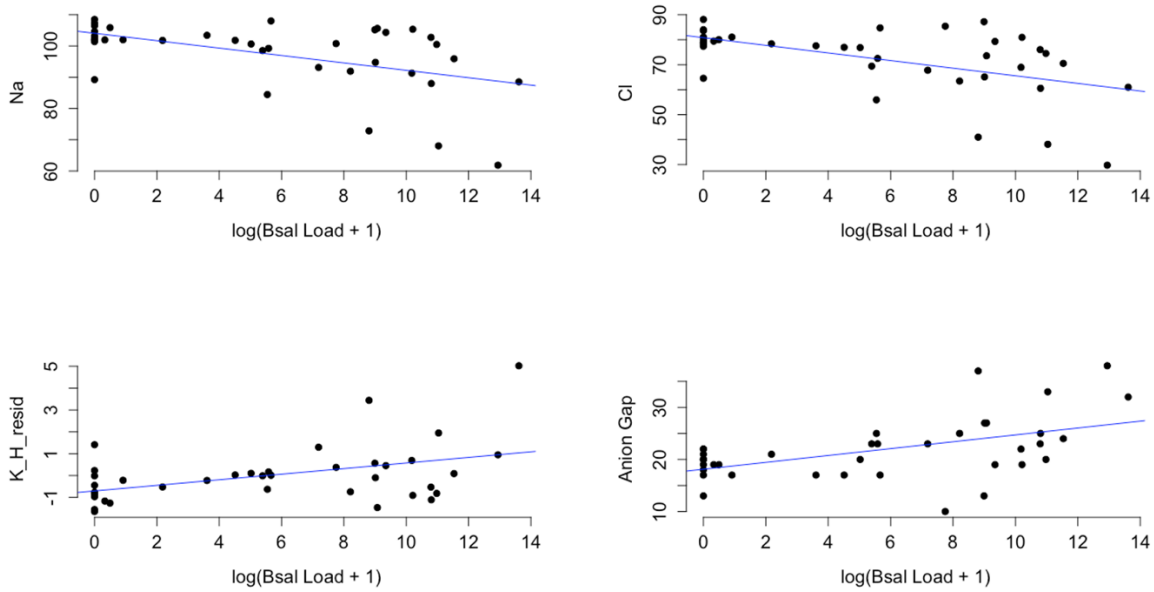

Figure 1.9 Plots of sodium (Na), chloride (Cl), the residuals of potassium (K) regressed on hemolytic index (H) (K_H_resid), and anion gap (y-axis) and the log of *Batrachochytrium salamandrivorans* qPCR load at time of necropsy + 1 (x-axis). Additional analysis showed that there was significant evidence for a non-linear quadratic effect of log(*Bsal* Load + 1) on chemistry values (log(*Bsal* Load + 1)^2 (effect from PERMANOVA: F=6.98, p=0.001).

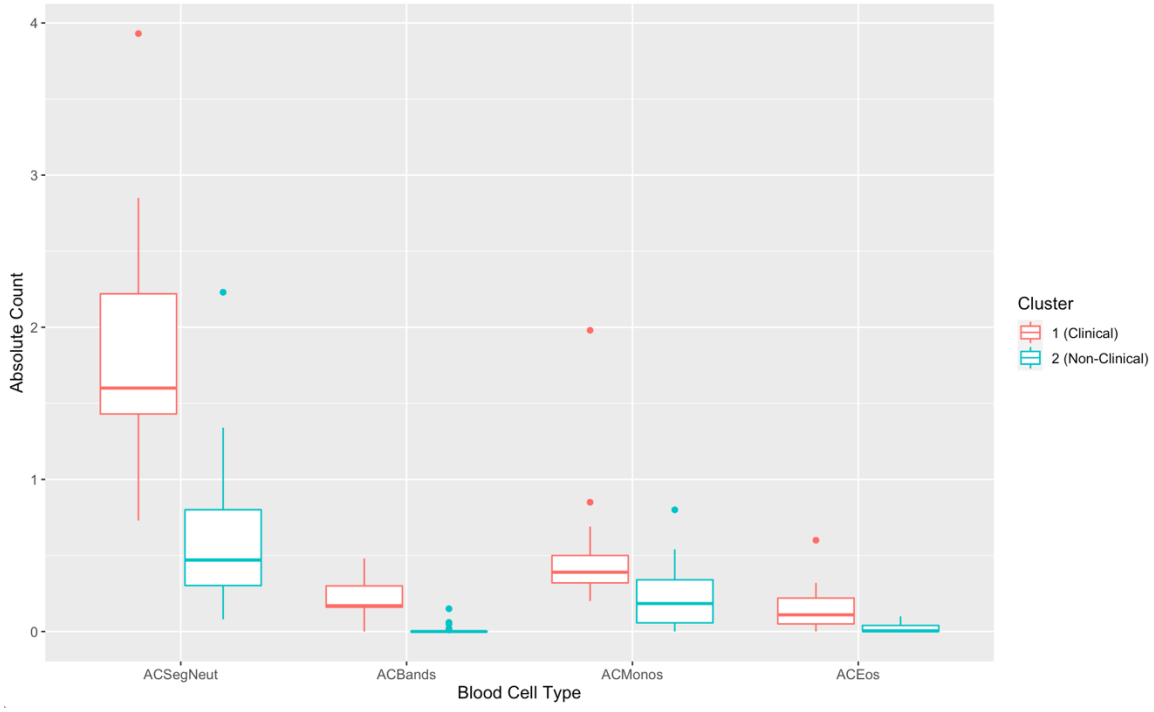

Figure 1.10 Boxplots comparing each complete blood count variable for cluster 1 (*Batrachochytrium salamandrivorans* (*Bsal*) infected, clinically diseased *Taricha granulosa*) in red and cluster 2 (controls and *Bsal*-infected, non-clinically diseased *T. granulosa*) in blue. Each box represents the interquartile range (IQR), the horizontal line within each box represents the median, vertical lines represent the 1.5 * IQR, and dots represent outliers. Absolute count of segmented neutrophils = ACSegNeuts; absolute count of band neutrophils = ACBands; absolute count of monocytes = ACMonos; absolute count of eosinophils = ACEos.

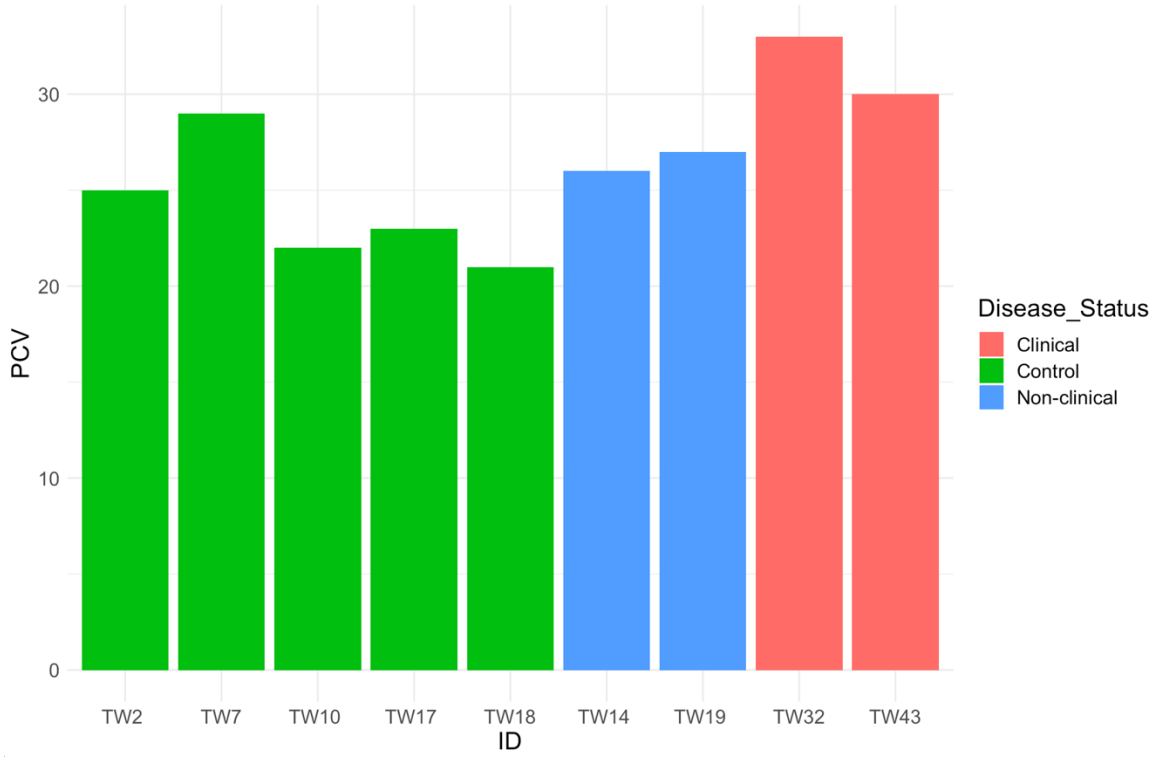

Figure 1.11 Packed cell volume for five control, two *Batrachochytrium salamandrivorans*-exposed/non-clinically diseased, and two *Batrachochtyrium salamandrivorans*- exposed/clinically diseased *Taricha granulosa*.

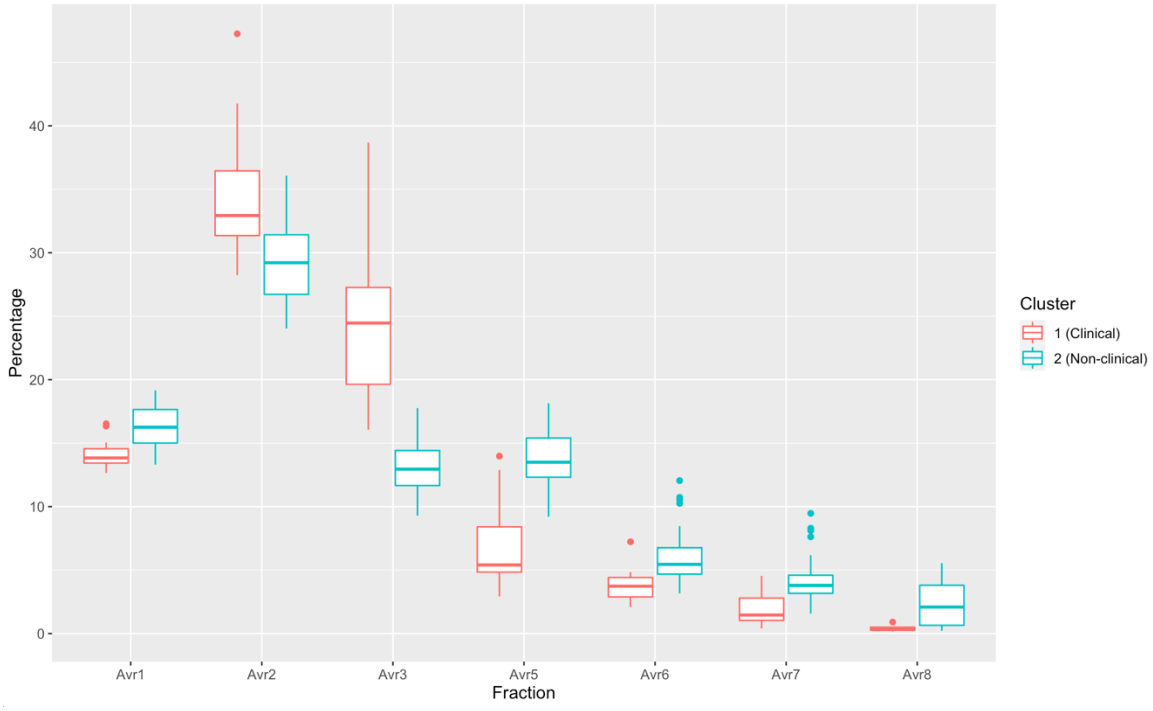

Figure 1.12 Boxplots comparing each protein electrophoresis fraction for cluster 1 (*Batrachochytrium salamandrivorans* (*Bsal*) infected, clinically diseased *Taricha granulosa*) in red and cluster 2 (controls and *Bsal*-infected, non-clinically diseased *T. granulosa*) in blue. Each box represents the interquartile range (IQR), the horizontal line within each box represents the median, vertical lines represent 1.5 * IQR, and dots represent outliers.

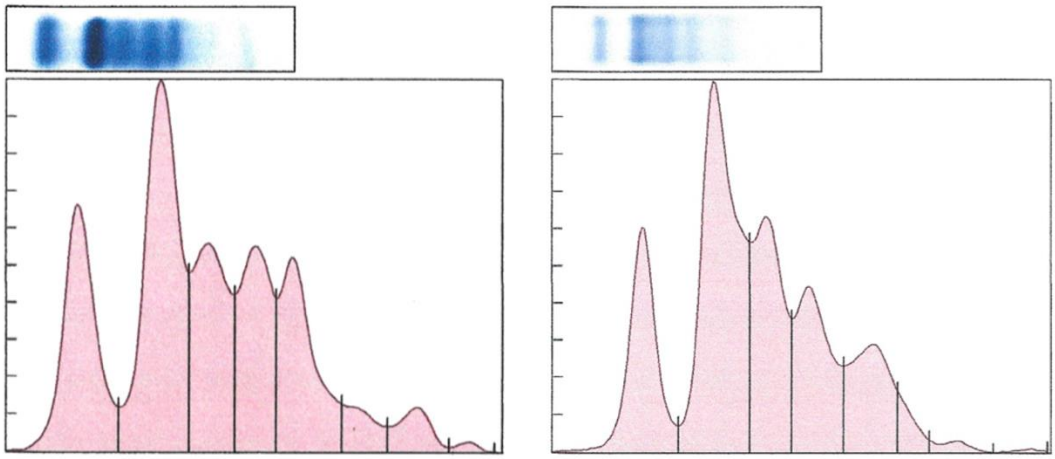

Figure 1.13 Representative protein electrophoretograms from a control (A) and a *Batrachochytrium salamandrivorans* infected, clinically diseased (B) *Taricha granulosa*.

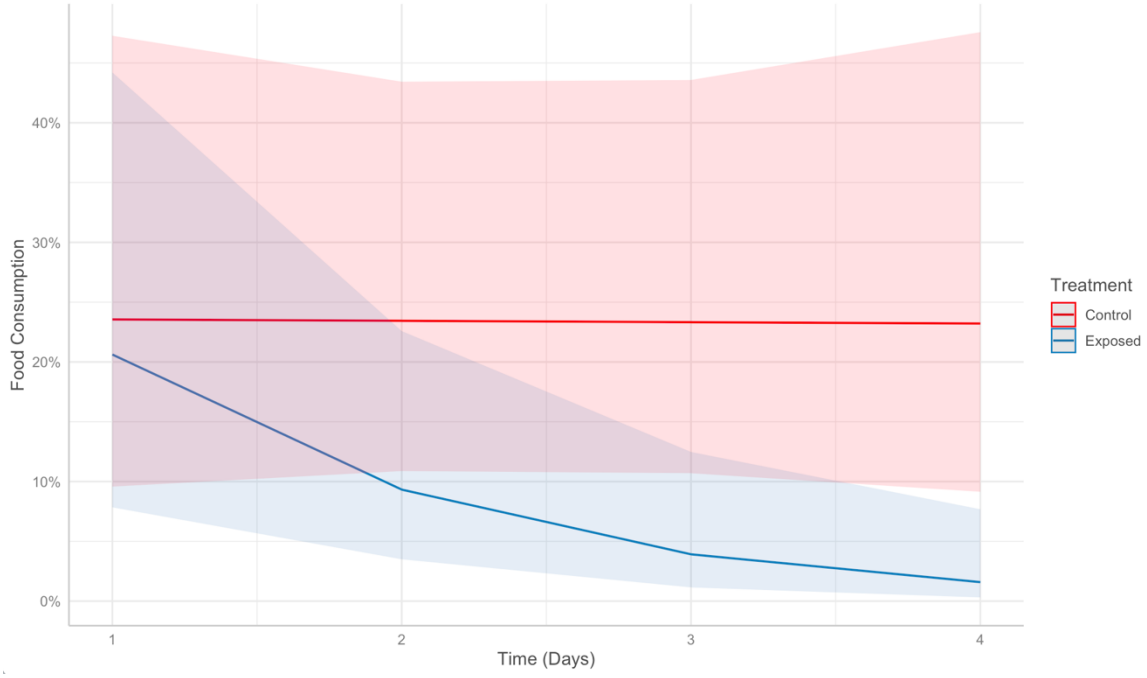

Figure 1.14 Food consumption over time (days) post-exposure in control (red) versus *Batrachochytrium salamandrivorans* exposed (blue) *Taricha granulosa*.

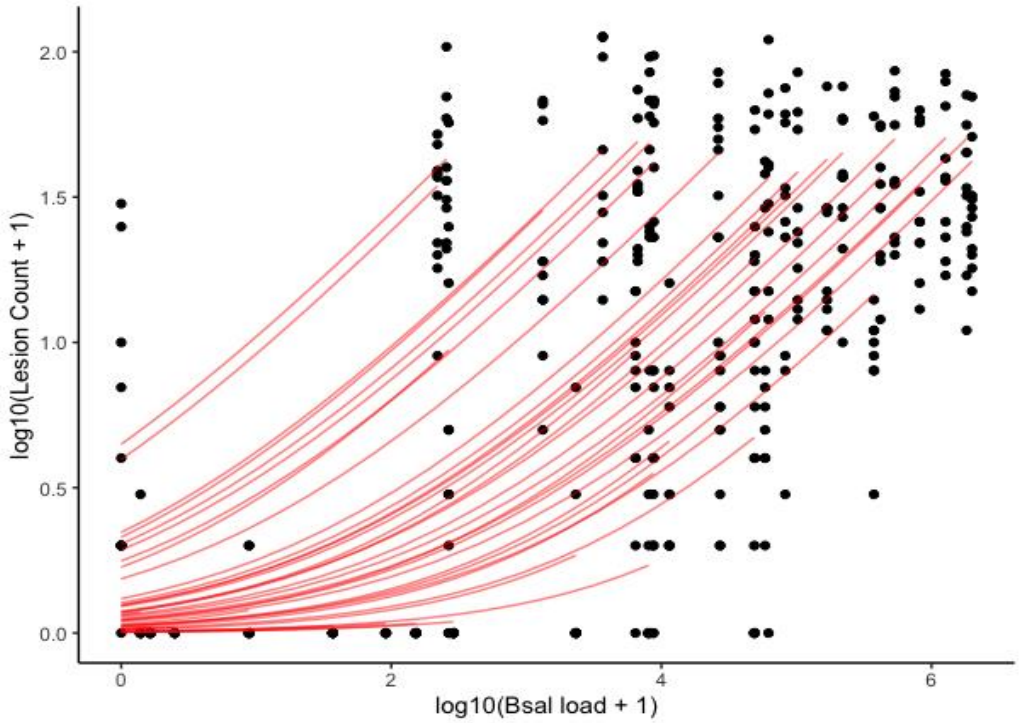

Figure 1.15 Plot of log10(Lesion count + 1) (y-axis) and log10(*Bsal* load + 1) (x-axis). Black points represent histologic lesion count at each of 10 standardized anatomical sections examined for each *Batrachochytrium salamandrivorans* (*Bsal*) infected *Taricha granulosa*. Red lines represent individual level relationships. As each individual only had one *Bsal* qPCR load (at necropsy), but multiple lesion counts due to the multiple sections, a unique slope cannot be inferred for the relationship between *Bsal* load and lesion count for each individual.

CHAPTER II: ENVIRONMENTAL TEMPERATURE AND PATHOGEN DOSE HAVE A SIGNIFICANT EFFECT ON HISTOLOGIC LESION COUNT AND SEVERITY IN *NOTOPHTHALMUS VIRIDESCENS* INFECTED WITH *BATRACHOCHYTRIUM SALAMANDRIVORANS*

Abstract

The pathogenic fungus, *Batrachochytrium salamandrivorans* (*Bsal*), was discovered a decade ago in Europe, where it is emerging and decimating salamander populations. North America is a global hotspot for salamander biodiversity and there is concern that *Bsal* could be introduced through trade or other pathways. One of the most abundant salamanders in eastern North America is the eastern newt (*Notophthalmus viridescens)*, and it is highly susceptible to *Bsal*. Previous studies suggest that eastern newts will play a major role in *Bsal* epidemiology if it is introduced, and environmental temperature may influence its invasion potential. What is less known is how environmental temperature contributes to *Bsal* pathogenesis, and the interaction between *Bsal* loads, skin lesion development, and morbidity. The goal of this study was to compare *Bsal*-induced lesions in eastern newts among four *Bsal* zoospore doses ($5x10^{3–6}$ per 10 mL) and maintained at three environmental temperatures (6, 14, and 22°C). Adult eastern newts were exposed to *Bsal* at one of the three target temperatures and chytridiomycosis was allowed to develop until either predetermined euthanasia endpoints were met, or the study period was completed. Following euthanasia, animals were preserved in 10% neutral buffered formalin, and standardized histology sections were taken across the body. *Bsal*-associated lesions were counted in each skin section, and lesions were graded for severity on a scale of 1–5. Additionally, dermal glands were examined for *Bsal* invasion, and all internal organs were assessed for abnormalities. Newts exposed at 22°C did not become infected by *Bsal*. I found that newts exposed at 14°C had increased lesion counts across all zoospore doses compared to those exposed at 6°C. For the lowest three zoospore doses, as zoospore dose increased, so did histologic lesion count. Additionally, there was a strong negative relationship between lesion count and survival, and the most common sections for histologic lesions overall were the hindlimbs, cloacal region, and tail. No significant abnormalities related to *Bsal* chytridiomycosis were identified in internal organs; further supporting the hypothesis that morbidity and mortality in infected individuals is directly related to skin lesions. These results provide insight into the pathogenesis of *Bsal* chytridiomycosis, and how environmental temperature can impact disease progression. Additionally, these results indicate swabbing the hindlimbs, cloacal region, and tail might increase detection of *Bsal* on infected animals due to increased lesion development at these locations.

1. Introduction

Emerging fungal diseases have recently caused unprecedented declines and extinctions in both plant and wildlife species (Fisher et al., 2012). Among affected wildlife species, amphibians have experienced the most devastating losses (Scheele et al., 2019). An emerging fungal disease involved in these declines is chytridiomycosis caused by two species of chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) (Scheele et al., 2019).

Bd was discovered in 1998 and is responsible for the majority of these declines (Berger et al., 1999; Yap et al., 2015). *Bsal,* which was more recently discovered in 2013, is more pathogenic to salamanders, and is quickly emerging (Scheele et al., 2019). It presumably originated in Asia and spread to Europe through the pet trade (Martel et al.,

2014; Yap et al., 2017), where it is currently causing large die-offs in multiple salamander species (Lotters et al., 2020; Martel et al., 2014; Martel et al., 2020).

Bsal has not yet been identified in North America (Waddle et al., 2020). However, due to multiple factors including environmental suitability, species susceptibility, and presence of *Bsal* in the pet trade, it is likely only a matter of time before *Bsal* spreads to regions other than Asia and Europe (Carter et al., 2021; Carter et al., 2019; Grant et al., 2017; Gray et al., 2015; Yap et al., 2017). We know little about how *Bsal* chytridiomycosis manifests and progresses in susceptible species under varying environmental conditions. Addressing this knowledge gap will inform disease identification in susceptible individuals and can be used to better understand the mechanisms of resistance and tolerance to this emerging pathogen.

Environmental temperature is especially important for amphibians as many physiologic functions, including the immune response, can be significantly affected by changes in temperature (Rollins-Smith, 2017, 2020; Wells, 2007). Previous studies have shown the pathogenicity of *Bsal* can change with changing temperatures in both eastern newts as well as fire salamanders (*Salamandra salamandra*) (Beukema et al., 2021; Blooi et al., 2015a; Carter et al., 2021; Stegen et al., 2017). Carter et al. evaluated the survival rate of *Bsal*-infected adult eastern newts across 6, 14, and 22°C and revealed a difference in survivability as well as *Bsal* qPCR load at time of necropsy between the three temperatures (Carter et al., 2021). However, no previous study has evaluated the number, distribution, and severity of histologic lesions in *Bsal*-infected hosts across multiple temperatures.

Incorporating histologic analysis of skin lesions in *Bsal* chytridiomycosis studies is imperative to fully understand the host-pathogen interaction (Thomas et al., 2018). This is important for two reasons. First, positive qPCR results alone do not confirm that infection with a pathogen is inducing disease. Some urodelan species are much more tolerant to *Bsal* infection than others (Wilber et al., 2021). Therefore, a *Bsal* qPCR load that corresponds with severe disease in one species or individual, may correspond with mild or no disease in another. Consequently, it is important to correlate qPCR results with histologic lesion analysis to determine disease causality and severity.

Second, it has been shown that enumeration of grossly visible skin lesions associated with *Bsal* infection in multiple urodelan species is not predictive of survival and only weakly positively correlated with *Bsal* qPCR load (Wilber et al., 2021). This may be because lesions are being missed without the use of histology. It has been shown that for *Bd*, qPCR-positive amphibians with no clinical signs or grossly apparent skin lesions can still have chytrid-induced skin lesions histologically (Borteiro et al., 2019). Therefore, incorporating histologic lesion counts and grades can provide a much better overall picture of disease progression in the animal than gross examination alone (Thomas et al., 2018).

A previous study investigating site predilection for *Bsal*-induced skin lesions did not incorporate lesion grade (Ossiboff et al., 2019). Higher lesion grade typically corresponds with increased numbers of intralesional *Bsal* organisms. Therefore, by incorporating lesion grade into histologic analysis, we can more effectively determine the

best anatomical locations to collect diagnostic samples in order to optimize pathogen detection in *Bsal*-infected amphibians.

Previous studies have investigated the effect of various *Bsal* zoospore doses on survival rate and determined that this pathogen causes dose-dependent mortality in multiple species (Carter et al., 2021; Carter et al., 2019). By replicating these studies with the addition of histologic lesion count, we can begin to elucidate how this pathogen leads to decreased survival rates in the individuals at higher doses. Overall, the aim of this study was to compare disease progression among multiple environmental temperatures and pathogen doses to better understand how *Bsal* causes morbidity in susceptible amphibian species.

2. Methods

Ethics Statement

Husbandry as well as euthanasia procedures followed recommendations provided by the American Veterinary Medical Association and the Association of Zoos and Aquariums. Additionally, they were approved by the University of Tennessee Institutional Animal Care and Use Committee (protocol #2623). *Taricha granulosa* that reached euthanasia endpoints were humanely euthanized via transdermal exposure to benzocaine hydrochloride.

Animals

A total of 75 *Notophthalmus viridescens* (eastern newts) were used in this study. Eastern newts were chosen as they have a wide distribution throughout North America (IUCN, 2020), and are known to be susceptible to *Bsal* chytridiomycosis (Carter et al., 2021; Longo et al., 2019; Martel et al., 2014). Animals were collected from Tennessee (TN Scientific Collection Permit #1504).

Animal care was similar to that of adult newts described by Carter, et al. (2021). At arrival into the laboratory, animals were heat-treated at a temperature of 30°C for 10 days to clear any potential *Bd* infection obtained in the wild (Bletz, 2013; Chatfield & Richards-Zawacki, 2011). After heat treatment, animals were skin swabbed following a standardized protocol of 10 swipes along the ventrum and 5 swipes along the plantar surface of each foot. DNA was extracted from the swabs, and *Bd* qPCR was performed following the protocol by Boyle et al. (2004). All animals were confirmed to be qPCR negative for *Bd* and underwent a one-week acclimation period to their assigned experimental temperature prior to the start of the study.

Animals were individually housed in sterilized, circular, 2000-cm^3, plastic containers filled with 300 mL of dechlorinated water along with a PVC cover object. Containers were changed and water replaced once every three days. Feeding took place every three days 24 hours prior to each water change and consisted of bloodworms (*Chironomus plumosus)* at 4% of their starting body weight. Individual containers were kept in an environmental growth chamber (Conviron, Winnipeg, Canada) set to their assigned temperature along with light/dark cycles which reflected the season

corresponding with the environmental temperature (winter=8 hours light; spring/autumn=10 hours light; summer=12 hours light).

Experimental Design

Experimental design was similar to that described by Carter et al. (2021). Animals were randomly assigned to one of four zoospore exposure doses ($5x10^3$, $5x10^4$, $5x10^5$, or $5x10^6$ per 10 mL) along with one of three temperatures (6, 14, and 22°C). Five animals were assigned to each treatment group along with five control animals (n=25 for each temperature trial). Animals were monitored twice daily for development of clinical signs and were euthanized when they reached humane disease endpoints (including loss of righting ability or unresponsiveness) or at the end of the study. Study duration was 60 days.

Fungal Culture

Bsal was isolated from a fire salamander (*Salamandra salamandra*) in the Netherlands and shared with my lab (isolate AMFP13/1, (Martel et al., 2013)). Cultures were maintained at the University of Tennessee Center for Wildlife Health laboratory and zoospores were enumerated according to previously described methods by Carter, et al. (2019).

Experimental Infection

Bsal-exposed individuals were exposed to either a dose of $5x10^3$, $5x10^4$, $5x10^5$, or $5x10^6$ zoospores per 10 mL by being individually placed into a 100-mL plastic tube containing a mixture of *Bsal* zoospores and 9-mL autoclaved dechlorinated water for 24 hours in the environmental incubators. Control animals were placed into 100-mL plastic tubes containing 10-mL of autoclaved dechlorinated water under the same time and environmental conditions as the exposed individuals.

Histopathology

Carcasses were stored in 10% neutral buffered formalin for a minimum of 48 hours prior to processing. Transverse sections through the entire body were taken at approximately 3-mm intervals and legs were removed at either the scapulohumeral or coxofemoral joint. Ten standardized sections were placed into tissue cassettes following the protocol listed in the appendix in Table 2.1 & Figure 2.1. Cassettes containing tissues were decalcified for 24 hours using a formic acid solution. Tissues were then routinely processed and stained with hematoxylin and eosin (H&E). Histologic examination consisted of examining all tissues for abnormalities as well as performing *Bsal* lesion counts and lesion grading in the skin for each of the 10 sections. Lesion grading consisted of scoring each lesion on a scale of 1–5 based on severity (Grade 1 = *Bsal* organisms within the stratum corneum, Grade 2 = *Bsal* organisms/associated cellular damage extending to the mid-epidermis, Grade 3 = *Bsal* organisms/associated cellular damage extending through the epidermis to the basement membrane, Grade 4 = coalescing/large *Bsal* lesions extending through the epidermis to the basement membrane which were <1 millimeter long, Grade 5 = coalescing/large *Bsal* lesions extending through the epidermis to the

basement membrane which were ≥1 millimeter long (Table 2.2 & Figure 2.2). Perimeter of each section was measured using Excelis Accu-Scope software to standardize measures of lesion counts to counts per unit area of cross section.

Quantitative Polymerase Chain Reaction (qPCR)

To detect *Bsal* and estimate loads, genomic DNA was extracted from each skin swab using the QIAamp 96 DNA QIAcube HT kit (Qiagen, Hilden, Germany) and qPCR performed similar to Blooi et al. (2015) using the Applied Biosystems Quantstudio 6 Flex qPCR instrument (Thermo Fisher Scientific Inc). All samples were run in duplicate and declared positive if both replicates reached cycle threshold prior to 50 amplification cycles (Carter et al., 2019).

Statistical Analyses

Lesion Counts

To determine how temperature and *Bsal* zoospore dose affected the number of lesions per histologic cross section, I fit a negative binomial generalized linear model where the response variable was lesion count per cross section perimeter and the predictor variables were temperature, *Bsal* zoospore dose, an interaction term between temperature and *Bsal* zoospore dose, and section number. Environmental temperature included 6°C and 14°C, *Bsal* zoospore dose included four levels $10^{3–6}$, and section number included sections 1–10, corresponding to different regions of the animal's body (Table 2.1 and Figure 2.1). I used a negative binomial distribution to describe lesion counts as initial analyses showed that lesion counts were over-dispersed relative to a Poisson distribution. In addition, lesion counts were obtained from histological cross sections with different perimeters, where a larger perimeter could lead to a higher number of counted lesions than a smaller perimeter even if the density of lesions per unit perimeter were the same. To account for this, I included an offset term of log(total perimeter) in the negative binomial regression (Faraway, 2016). I fit the lesion count model using the brms package in R (Burkner, 2017), with a weakly regularizing prior on the effect sizes for the predictor variables temperature, *Bsal* zoospore dose, and section number. When fitting the models, I ensured all convergence by visually examining chains and checked that R_hat (the potential scale reduction factor) < 1.01 and effective sample size > 400 for all parameters (Vehtari et al., 2020). I fit five candidate models to the data and selected the best predictive model using Pareto smoothed importance sampling leave-one-out information (PSIS-LOO, (Vehtari et al., 2016). Models with lower PSIS-LOO indicate better predictive performance. I compared models including the following combinations of predictors to my final model described above: 1) temperature and *Bsal* zoospore dose, 2) temperature, *Bsal* zoospore dose, and temperature and *Bsal* zoospore dose as an interaction term, and 3) temperature, *Bsal* zoospore dose, and section number.

Probability of Lesion Presence

To determine how temperature and *Bsal* zoospore dose affected the probability of lesion presence, I fit a Bernoulli generalized linear model where the response variable was lesion presence and the predictor variables were *Bsal* zoospore dose, temperature, an

interaction term between *Bsal* zoospore dose and temperature, and the scaled log(perimeter) as a covariate rather than an offset term in this analysis. However, the log(perimeter) covariate effectively acted as an offset, accounting for the fact that larger cross sections had a higher probability of lesion presence, all else being equal. I used a Bernoulli distribution to describe lesion presence as I was interested in the probability of a lesion being present at each *Bsal* zoospore dose/temperature treatment combination. I fit the model using the brms package in R (Burkner, 2017), with a weakly regularizing prior on the effect sizes for the predictor variables temperature, *Bsal* zoospore dose, and perimeter. When fitting the models, I ensured model convergence using the previously described methods. I fit four candidate models to the data and selected the best predictive model using PSIS-LOO. I compared models including the following combinations of predictors to my final model described above: 1) the scaled log(perimeter), 2) *Bsal* zoospore dose, temperature, and scaled log (perimeter), 3) *Bsal* zoospore dose, temperature, *Bsal* zoospore dose and temperature as an interaction term, scaled log(perimeter), and section number.

Lesion Grades

To determine how temperature and *Bsal* zoospore dose affected the expected lesion density of each lesion grade per unit cross section, I fit a generalized linear model where the response variable was lesion count, and the predictor variables were lesion grade, the interaction term of lesion grade and temperature, the interaction term of lesion grade and *Bsal* zoospore dose, the interaction term of lesion grade and section number, and the random effect of animal ID. Lesion grade consisted of lesion grades 1–5. This model was an extension of a multinomial regression model where lesion grades were the multinomial categories, re-expressed as a Poisson or negative binomial log-linear model (Faraway, 2016). I originally included lesion grades 1–5 in the model; however, grade 5 lesions were dropped from the analysis due to their relative rarity leading to issues with separability. I fit models with both a Poisson and negative binomial distribution of lesion counts, and the negative binomial was preferred due to the overdispersion of lesion counts. Additionally, I included an offset term of log(total perimeter) in the log-linear regression as described in the lesion count analysis.

I fit the model using the brms package in R (Burkner, 2017), with a weakly regularizing prior on the effect sizes for the predictor variables temperature, *Bsal* zoospore dose, and lesion grade ([prior = 0,3]; all continuous covariates were standardized to a mean of 0 and a standard deviation of 1 before fitting). When fitting the models, I ensured model convergence using the previously described methods. I fit eight candidate models to the data and selected the best predictive model using PSIS-LOO. I compared models including the following combinations of predictors (using both a negative binomial distribution and a Poisson distribution for each) to my final model described above: 1) lesion grade and individual ID as a random effect, 2) lesion grade, lesion grade and temperature as an interaction term, lesion grade and *Bsal* zoospore dose as an interaction term, and individual ID as a random effect, 3) lesion grade, lesion grade and temperature as an interaction term, lesion grade and *Bsal* zoospore dose as an interaction term, the combination of lesion grade, temperature, and *Bsal* zoospore dose as

an interaction term, and individual ID as a random effect. For potentially influential observations with a Pareto smoothing k > 0.7, I temporarily excluded these values to see if they affected my model selection. My model selection was unaffected by these potentially influential observations, and they were included in our model inference. Finally, *Bsal* zoospore dose was replaced with *Bsal* qPCR load at the time of necropsy in all models and were compared to original models using PSIS-LOO.

Survival Analysis

To determine how *Bsal* zoospore dose and lesion count affected survival rate, I fit a Cox proportional hazards model where the response variable was days survived and the predictor variables were *Bsal* zoospore dose and lesion count, where lesion count was transformed as log(lesion count + 1). In my experiment, some of the *N. viridescens* were euthanized and others died. I considered euthanized individuals as right-censored. When fitting the models, I ensured the proportional hazards assumption was met by confirming a non-significant relationship between Schoenfeld residuals for each covariate and time (Klein & Moeschberger, 2003). I checked overall goodness-of-fit by plotting cumulative hazard against Cox-Snell residuals and visually assessed for clear outliers by plotting deviance residuals against observation number. Additionally, I compared models including *Bsal* zoospore dose to models including *Bsal* qPCR load at the time of necropsy using Akaike information criteria (AIC), where lower AIC indicates better predictive performance. Models including *Bsal* zoospore dose were determined to be a better predictor of survival. All models were fit with using the coxph function in the `survival` package in R (version 4.1.1, (Therneau & Lumley, 2015).

3. Results

Lesion Counts

Across all *Bsal* zoospore doses, *N. viridescens* exposed at 14°C had a higher expected lesion count per average cross section than those exposed at 6°C (Fig. 2.3), though the effect of temperature changed with zoospore dose (Difference in PSIS-LOO for models with and without the temperature by zoospore dose interaction = 41.2; Fig. 2.3). *Notophthalmus viridescens* exposed to the 5 x 10^5 zoospore dose at 14°C had the overall highest expected lesion count per average cross section (Expected number of lesions per average cross section for the 10^5/14°C treatment: E: 77.24, 95%CI: 55.70–109.15; Fig 2.3). Due to the significant interaction between temperature and zoospore dose, individuals exposed to the 5 x 10^6 zoospore dose at 14°C had lower expected lesion counts than those at 5 x 10^5 doses (Difference between 10^5 and 10^6 at 14°C: E: 41.92, 95%CI: 22.31–69.78; Fig. 2.3). Across all zoospore dose and temperature combinations, sections 6 (hindlimb), 9 (cloacal region) and 10 (tail) had the highest expected lesion counts relative to the baseline of Section 1 (effect sizes relative to Section 1: [(section 6 = E: 0.57; 95%CI: 0.2–0.95); (section 9 = E: 0.57; 95%CI: 0.18–0.98); (section 10 = E: 0.85; 95%CI: 0.48–1.23)]; Fig. 2.3).

Probability of Lesion Presence

Notophthalmus viridescens exposed to the two lowest zoospore doses had a greater probability of having *Bsal*-associated skin lesions at 14°C than at 6°C (Estimate of difference between the probability of lesion presence at 14°C minus the probability of lesion presence at 6°C for 10^3: E: 0.62, 95% CI: 0.46–0.75; Estimate of difference between the probability of lesion presence at 14°C minus the probability of lesion presence at 6°C for 10^4: E: 0.15, 95%CI: 0.07–0.27; Fig. 2.4). *Notophthalmus viridescens* exposed to the two highest zoospore doses had an approximately equal probability of having *Bsal*-associated skin lesions regardless of temperature (95% CI of difference between the probability of lesion presence at 14°C minus the probability of lesion presence at 6°C for 10^5: -0.02–0.03; [95% CI of difference between the probability of lesion presence at 14°C minus the probability of lesion presence at 6°C at 10^6: -0.02–0.02]; Fig. 2.4). Finally, *N. viridescens* exposed to zoospore doses $5 \times 10^{4-6}$ were more likely to have lesions present than those exposed to a zoospore dose of 5×10^3 regardless of the temperature (Fig. 2.4).

Lesion Grade

Regardless of temperature, I found that Grade 1 lesions were the most common at the lowest zoospore dose, Grade 3 lesions were the most common at the 5×10^4 and 5×10^6 zoospore doses, and Grade 2 lesions were the most common at the 5×10^5 zoospore dose (Fig. 2.5). Grade 5 lesions were the least common grade of lesion across all zoospore dose/temperature combinations (Total number of grade 5 lesions observed from *N. viridescens* across all zoospore dose/temperature combinations = 83). Grade 4 lesions were the second least common grade of lesion across all zoospore dose/temperature combinations (Fig. 2.5). Similar to my analysis on overall lesion counts, section number (sections 6 and 10) was an important predictor of lesion grade, particularly for Grade 4 and Grade 5 lesions (Difference in PSIS-LOO for models with and without Section as a factor = 579.9). As determined previously, temperature also had a significant effect on lesion count; however, the magnitude of the effect varied with lesion grade (Fig. 2.5).

Survival Analysis

A strong negative relationship was identified between lesion count and survival duration, with increasing lesion count correlating with decreased survival (Hazard ratio: 1.65; 95%CI: 1.46-1.86 for the lesion count effect). This strong relationship persisted after accounting for either *Bsal* zoospore dose or *Bsal* infection intensity at necropsy, indicating that lesions were describing variability in host survival beyond exposure or infection intensity (Table 2.3). *Notophthalmus viridescens* exposed to the 5×10^6 zoospore dose had the shortest survival time followed by 5×10^5, 5×10^3, and 5×10^4 zoospore doses (Fig. 2.6).

Internal Organ and Dermal Gland Examination

No lesions associated with *Bsal* chytridiomycosis were identified in internal organs. The only abnormalities identified in both control and exposed individuals included variable amounts of melanomacrophage hyperplasia in the liver as well as various parasites within

the coelomic cavity, gastrointestinal tract, and skeletal muscle. Parasites included mesomycetozoans, trematodes, nematodes, and cestodes, and were associated with minimal to no inflammatory response. For the 6°C animals, dermal gland invasion was identified in 1–2 dermal glands in one 10^3, one 10^4, two 10^5, and one 10^6 individual(s). For the 14°C animals, dermal gland invasion was identified in 1–4 dermal glands in two 10^4, one 10^5, and two 10^6 individual(s). All areas of dermal gland invasion had *Bsal* organisms and associated cellular damage in the overlying dermis and epidermis which was variably eroded to ulcerated.

4. Discussion

The main objective of this study was to determine how environmental temperature and pathogen dose relate to disease progression in *Bsal*-exposed *N. viridescens*. Environmental temperature as well as *Bsal* zoospore exposure dose were both shown to have a significant effect on histologic lesion development. *Notophthalmus viridescens* exposed at 22°C did not become infected at any *Bsal* zoospore dose. *Notophthalmus viridescens* exposed at 14°C had increased lesion counts compared to those exposed at 6°C across all zoospore doses. These findings support those from a previous study, which determined that *N. viridescens* exposed to *Bsal* at 14°C had overall lower survival rates than those exposed at 6°C or 22°C (Carter et al., 2021). Lower survival rates in environmental conditions associated with higher lesion counts supports the hypothesis of *Bsal* chytridiomycosis having a similar pathogenesis to *Bd* chytridiomycosis, involving pathogen-induced epithelial damage being the primary cause of morbidity and mortality in infected individuals (Voyles et al., 2009).

The reason for these differences between exposure temperatures is likely multifactorial and due to variations in both the host response to infection and the pathogenicity of the fungus. Carter et al. (2021) showed that *N. viridescens* exposed at 6°C tend to survive longer and with a lower *Bsal* qPCR load at necropsy than those exposed at 14°C, likely indicating a decreased infection tolerance at this temperature. A similar trend was also identified in a separate study involving *S. salamandra* (Stegen et al., 2017). In my study, histologic lesion count at the time of death/euthanasia was lower in 6°C animals than in 14°C animals, supporting the theory of decreased infection tolerance at this temperature (i.e. animals are dying with fewer lesions). This may be due in part to a decreased immune response to the pathogen, as colder temperatures have been shown to lead to decreased immune system function in amphibians (Rollins-Smith, 2017).

Carter et al. (2021) identified another potential method of host response to *Bsal* infection in that *N. viridescens* housed at 6°C had a larger amount of total recovered proteins on their skin than those housed at 14°C. They proposed that these proteins may have inhibitory effects against *Bsal* zoospores which could have led to decreased rate of disease progression in the 6°C animals. Decreased histologic lesion counts at this temperature compared to at 14°C also support this theory. By incorporating histologic lesion counts in my study, I have gained an understanding of disease progression, which Carter et al. (2021) was not able to assess without the use of histology.

In regard to changes in the pathogenicity of the fungus itself, it has been determined that the optimal temperature for maximum growth of *Bsal in vitro* is between 10°C and 15°C, with reduced growth at lower temperatures (Martel et al., 2013). Therefore, at 6°C, *Bsal* replication rate may have been reduced compared to at 14°C. This may have also contributed to decreased lesion numbers on *N. viridescens* exposed at this temperature. Previous studies have shown that *S. salamandra* housed at 25°C can clear *Bsal* infection with heat treatment alone, (Blooi et al., 2015a) and at 20°C in combination with antibiotic and antifungal agents (Blooi et al., 2015b). This study as well as a study by Carter et al. (2021) also confirmed no infection when *N. viridescens* were exposed to *Bsal* at 22°C.

The probability of lesion presence being equal between 6°C and 14°C at the two highest *Bsal* zoospore doses indicates that when *N. viridescens* are exposed to a high enough load of the pathogen, they will become infected regardless of the environmental temperature at 6 or 14°C. This is supported by the decreased survival rate seen in the two highest zoospore doses in this study. Additionally, similar survival rate findings were reported in a previous study (Carter et al., 2021).

Across all 6°C and 14°C temperature and zoospore dose combinations, grade 5 lesions were the rarest, followed by grade 4 lesions. These lesion grades occurred most at the higher zoospore doses in the 14°C temperature group. As these are the most severe lesion grades, it is likely that once lesions this severe begin to develop, mortality occurs shortly after.

The hindlimbs, cloacal region, and tail were identified as the most common sites for lesion development across all 6°C and 14°C temperature and zoospore dose combinations. Additionally, the hindlimbs and tail were where the most severe lesions occurred. The hindlimbs and tail were also identified to be predilection sites for *Bsal*-associated lesions histologically in a previous study in *N. viridescens*; however, this study did not investigate lesion grade (Ossiboff et al., 2019). This information is very important for disease surveillance efforts as it provides knowledge regarding the best anatomic location to collect diagnostic samples to obtain the highest chance of detecting the pathogen in *Bsal*-infected animals. Additionally, these sections are of particular importance when examining an animal grossly for *Bsal*-associated skin lesions. Since these sites have the most numerous and most severe lesions, they are more likely to be visible without microscopic examination. It is important to note that additional studies investigating *Bsal* skin lesion distribution in naturally infected animals should be performed as exposure method could affect lesion distribution in experimental studies.

At 14°C, *N. viridescens* exposed to the second highest zoospore dose had the greatest expected lesion density per cross section. This is an interesting finding as the highest zoospore dose might be expected to have the highest lesion count. However, the reasoning for this is that although more lesions were present in animals at the 10^5 dose, the lesions were more commonly less severe than those present in the 10^6 animals. Therefore, the 10^6 animals had a lower expected lesion density with overall more severe lesions.

Histologic lesion count was determined to have a strong negative correlation with survival time after accounting for *Bsal* infection intensity, with increasing lesion counts

leading to decreased probability of survival. A previous study assessing gross rather than histologic lesion count showed that lesion count was not predictive of survival (Wilber et al., 2021). This highlights the importance of incorporating histology into *Bsal* chytridiomycosis studies to fully understand the impact of infection (Thomas et al., 2018).

Invasion of dermal glands with *Bsal* zoosporangia was identified in at least one *N. viridescens* from the majority of zoospore dose treatments within the 6°C and 14°C treatment groups. Amphibians possess two types of glands within their dermis classified as either granular glands or mucous glands. These glands have many important functions including maintaining moisture and permeability of the skin, producing and secreting antimicrobial peptides as a key component of the innate immune response, and producing substances to deter predators (Varga et al., 2018). Infection of dermal glands in *Bsal* chytridiomycosis has been reported previously (Ossiboff et al., 2019), and has been proposed as a potential unique component of *Bsal* pathogenesis (Chapter I), as this does not occur in cases of *Bd* chytridiomycosis (Berger et al., 2005; Longcore et al., 1999). Carter et al. (2021) found that at two months post *Bsal* exposure, *N. viridescens* exposed at 6°C had significantly less defensive hydrophobic molecules produced through skin secretions than control *N. viridescens*, which could also be supportive of damage to these glands being an aspect of disease pathogenesis (Carter et al., 2021). Secretion of these defensive molecules has also been shown to be associated with stress as well as other types of injury and infection (Varga et al., 2018). Therefore, future studies assessing impaired function of these glands associated with *Bsal* infection are warranted.

As in other chytridiomycosis studies, no internal lesions directly related to chytridiomycosis were identified. Varying amounts of melanomacrophage hyperplasia were noted within the liver of both control and exposed *N. viridescens*; however, this is a non-specific finding. Multiple types of parasites were documented within the coelomic cavity, gastrointestinal tract, and skeletal muscle; however, they incited no to minimal inflammation and were considered incidental. Lack of internal lesions provides further support to the proposed pathogenesis of chytridiomycosis primarily involving damage to the skin cells leading to electrolyte imbalances and cardiac arrest (Voyles et al., 2009). Death associated with electrolyte imbalances is often very acute, and therefore, histologic lesions frequently do not have time to develop in internal organs such as the heart.

Overall, findings confirm that histologic lesion count and grade are influenced by both environmental temperature as well as zoospore dose in *Bsal*-infected *N. viridescens*. Additionally, my results provide support for the pathogenesis of chytridiomycosis primarily involving damage to the epidermal cells (Chapter I). I also provide further evidence that dermal gland invasion could potentially play a role in *Bsal* chytridiomycosis disease pathogenesis (Ossiboff et al., 2019), as invasion of glands was identified in multiple individuals across treatment groups.

Future studies should assess the effect of temperature and zoospore dose on histologic lesion count in additional amphibian species, as each species responds differently to infection (Martel et al., 2014). This will further our knowledge of host-pathogen interactions as well as increase our understanding of pathogen tolerance (Wilber et al., 2021). Additionally, future studies incorporating other diagnostic methods such as

complete blood cell counts, blood gas evaluation, and plasma protein electrophoresis would be useful to better understand what disturbances are occurring in the host and leading to morbidity and mortality associated with skin lesions (Chapter I).

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APPENDICES

Appendix A: Tables

Table 2.1 Anatomic location associated with each of ten standardized sections examined histologically for lesion counts and grading in *Batrachochytrium salamandrivorans* infected *Notophthalmus viridescens.*

Section Number	Anatomic Location
1	Cranial to eye
2	Caudal to eye
3	Cranial to thoracic limbs
4	Caudal to thoracic limbs
5	Left forelimb
6	Right hindlimb
7	Mid-body
8	Cranial to hindlimbs
9	Caudal to hindlimbs
10	Caudal 3 cm of the tail

Table 2.2 Description of histologic lesion grading system (1–5) used in *Batrachochytrium salamandrivorans* infected *Notophthalmus viridescens*.

Lesion Grade	Description
1	Lesion/Organisms extending into the stratum corneum only
2	Lesion/organisms extending to the level of the mid-stratum spinosum
3	Lesion/organisms extending through the full thickness of the epidermis
4	Coalescing/large lesion which extends full thickness of the epidermis and is <1 millimeter in length
5	Coalescing/large lesion which extends full thickness of the epidermis and is ≥ 1 millimeter in length

Table 2.3 Akaike information criterion (AIC) for candidate statistical models included in survival analysis in *Notophthalmus viridescens* exposed to one of four *Bsal* zoospore doses.

Statistical Model	AIC
Days Survived ~ Lesion count	1482.70
Days survived ~ Lesion count + Bsal load at necropsy	1463.86
Days survived ~ Lesion count + Bsal zoospore dose	1440.03

Appendix B: Figures

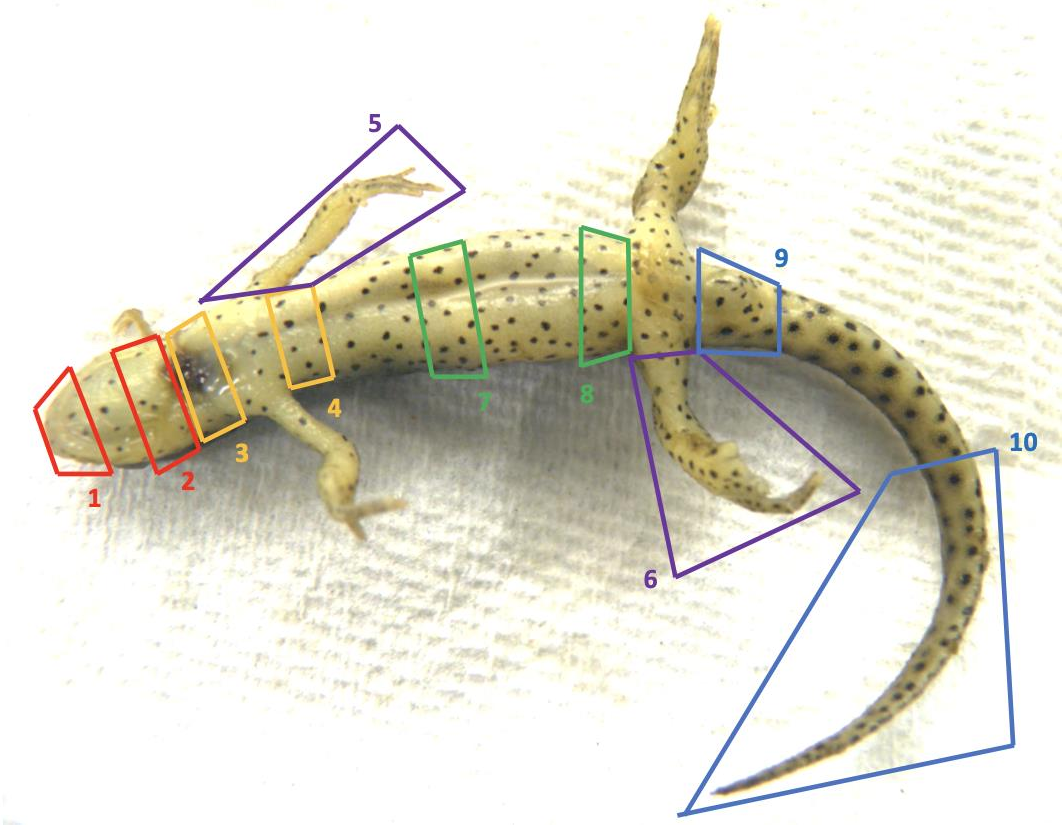

Figure 2.1 Anatomic location associated with each of the ten standardized sections examined histologically for lesion counts and grading in *Batrachochytrium salamandrivorans* infected *Notophthalmus viridescens*.

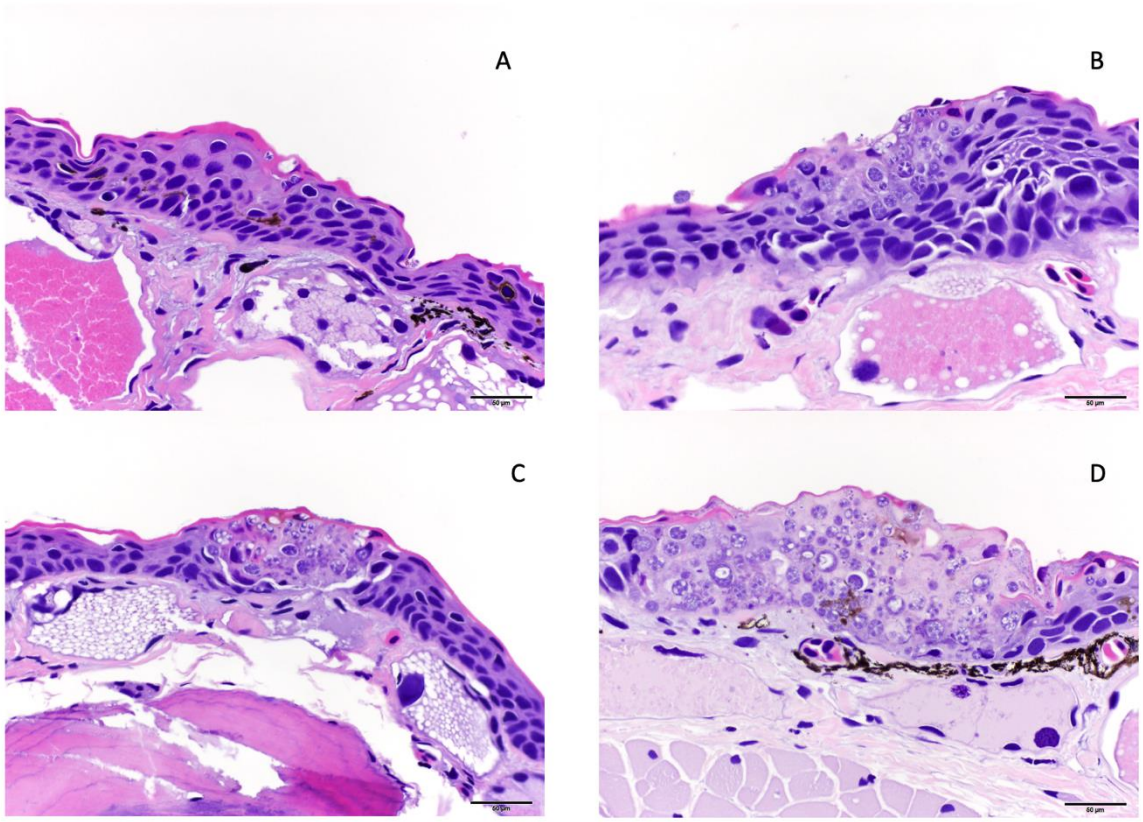

Figure 2.2 Examples of histologic lesion grading scheme used for *Notophthalmus viridescens* infected with *Batrachochytrium salamandrivorans* (*Bsal*) including Grade 1 (A), Grade 2 (B), Grade 3 (C), and Grade 4 (D). Grade 1 lesions were limited to *Bsal* organisms within the stratum corneum, grade 2 lesions included *Bsal* organisms and associated cellular damage which extended into the mid-epidermis, grade 3 lesions included *Bsal* organisms and associated cellular damage which extended full thickness through the epidermis, grade 4 lesions included coalescing/large lesions in which *Bsal* organisms and associated cellular damage extended full thickness through the epidermis and were <1 millimeter in length.

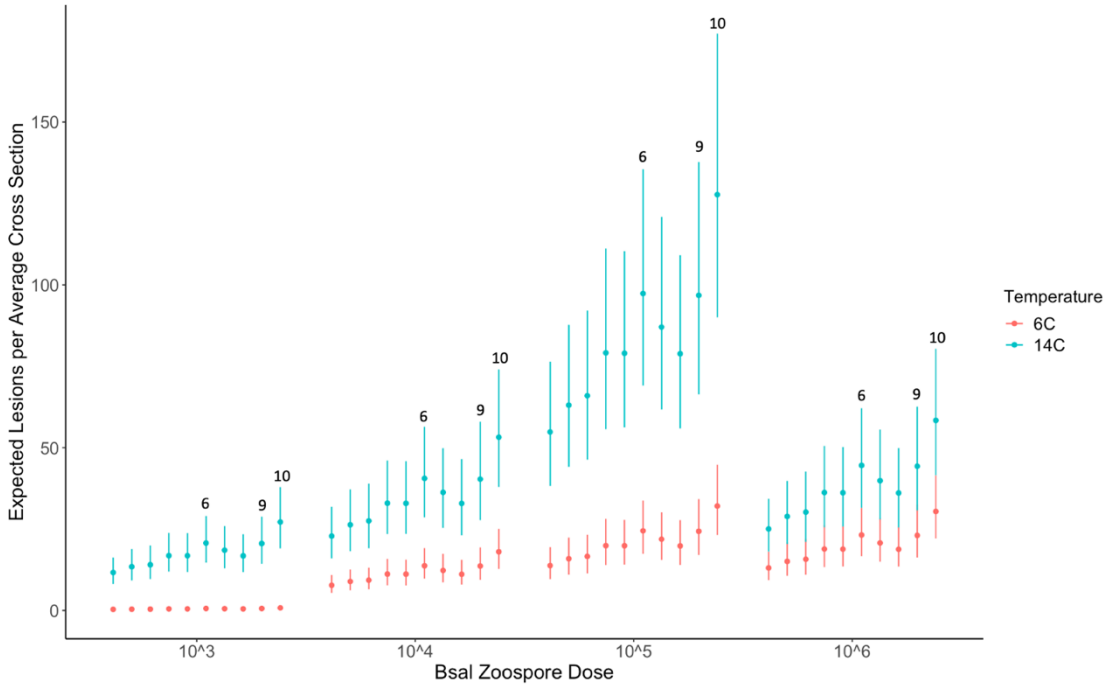

Figure 2.3 Plot of the expected histologic lesions per average cross section (y-axis) for each *Batrachochytrium salamandrivorans* (*Bsal*) zoospore dose (x-axis) and temperature (6°C = red; 14°C = blue) treatment combination in *Bsal*-infected *Notophthalmus viridescens*. Each point within a zoospore dose represents a section in numerical order from section 1–section 10. Error bars are showing the 95% confidence intervals around the predicted response.

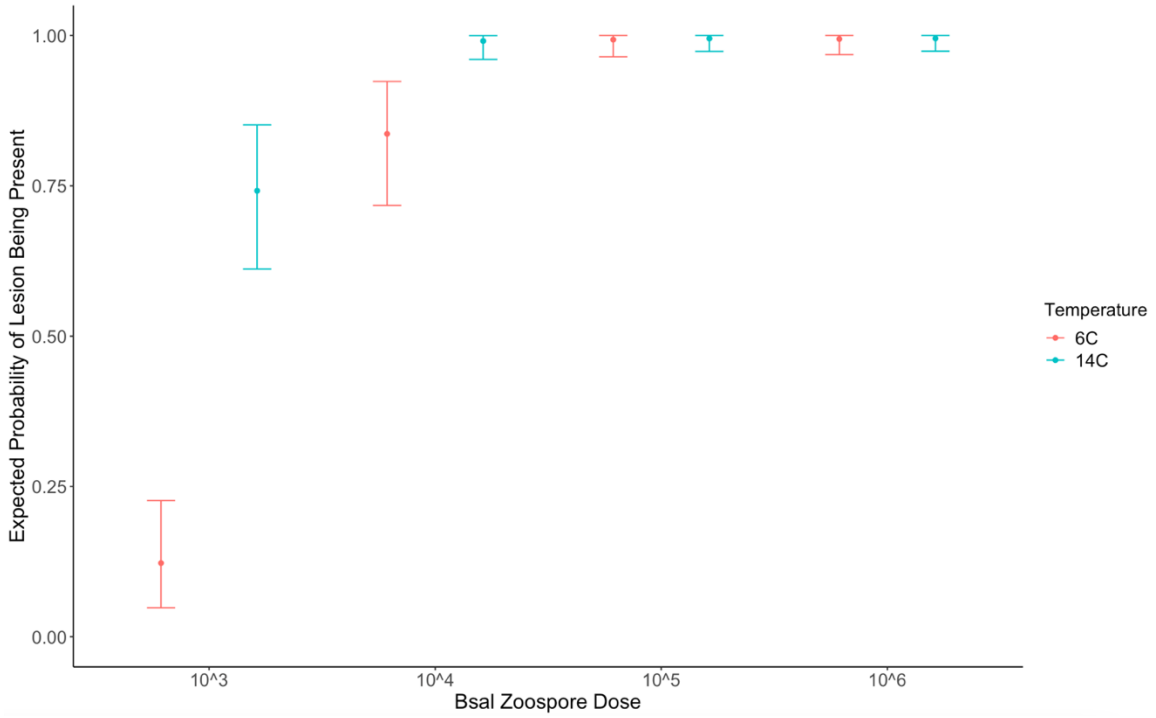

Figure 2.4 Plot of the expected probability of histologic lesions being present (y-axis) across each *Batrachochytrium salamandrivorans* (*Bsal*) zoospore dose (x-axis) and temperature (6°C = red; 14°C = blue) treatment combination in *Bsal*-infected *Notophthalmus viridescens*. Error bars are showing the 95% confidence intervals around the predicted response.

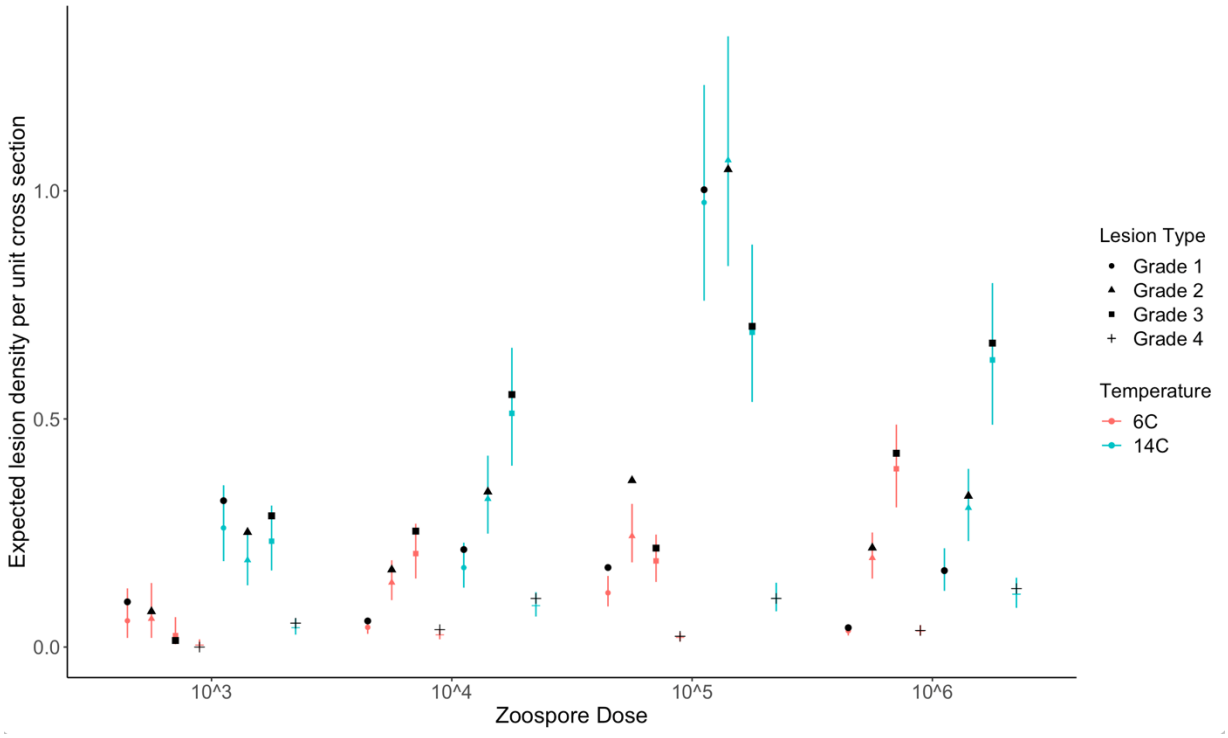

Figure 2.5 Plot of the expected histologic lesion density per unit of cross section (y-axis) for each *Batrachochytrium salamandrivorans* (*Bsal*) zoospore dose (x-axis) and temperature (6°C = red; 14°C = blue) treatment combination in *Bsal*-infected *Notophthalmus viridescens*. Each point within a zoospore dose represents a lesion grade in numerical order from Grade 1–Grade 4 along with an associated shape (Grade 1 = circle; Grade 2 = triangle; Grade 3 = square; Grade 4 = +). Black points represent the observed mean lesion density for each lesion grade within a zoospore dose and temperature. Error bars are showing the 95% confidence intervals around the predicted response.

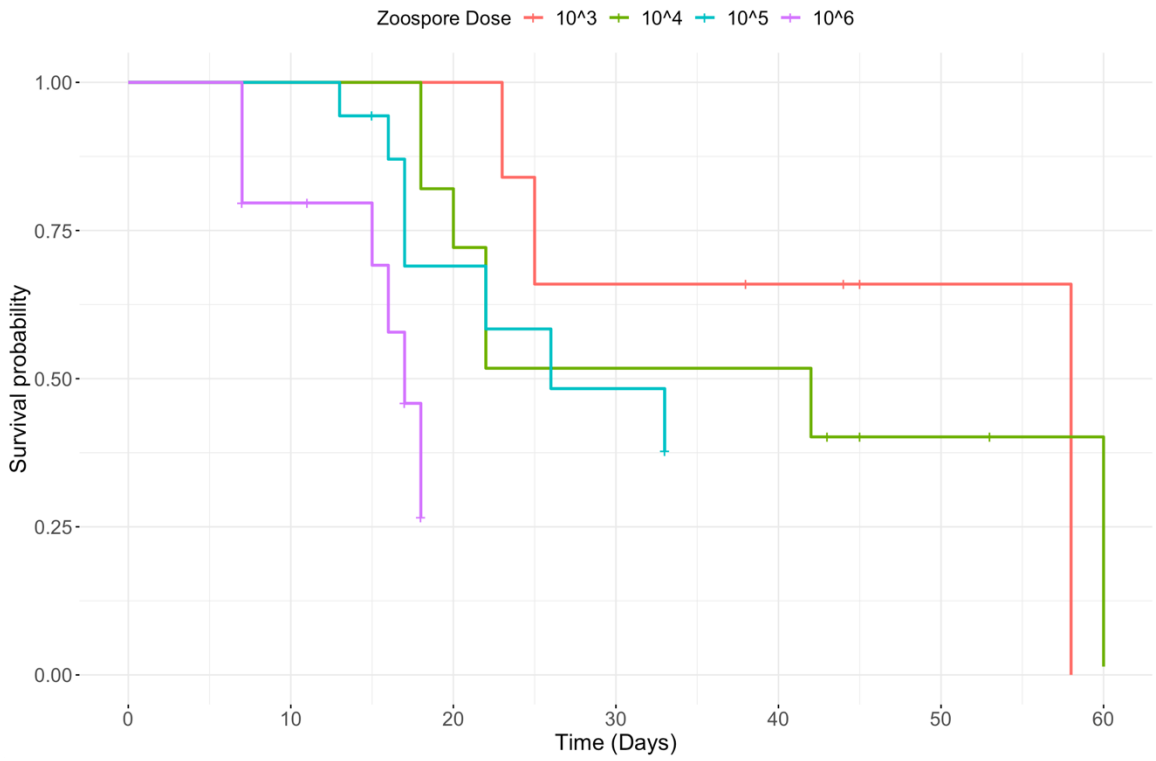

Figure 2.6 Survival curves representing the mortality rate of *Notophthalmus viridescens* exposed to one of four *Batrachochytrium salamandrivorans* zoospore doses (5 x $10^{3–6}$).

CHAPTER III: PLASMA PROTEIN ELECTROPHORESIS IN ONE ANURAN AND SIX URODELAN SPECIES

Abstract

As threats to amphibian health increase, there is an increasing need for diagnostic tools to assess and monitor their health status. One such tool which has undergone very little investigation in amphibians but has proven to be useful in other non-mammalian species is plasma protein electrophoresis. For this diagnostic tool to be useful in amphibians, we must first define the amphibian electrophoretogram and then obtain baseline reference intervals across species. I aim to provide representative plasma protein electrophoretograms for clinically normal amphibian(s) of seven different species, which have not been described previously. Blood samples were collected immediately postmortem by cardiocentesis from clinically normal individuals of each of the following species: *Osteopilus septentrionalis* (Cuban tree frog, n=2), *Gyrinophilus porphyriticus* (spring salamander, n=1), *Notophthalmus viridescens* (eastern newt, n=1), *Eurycea guttolineata* (three-lined salamander, n=2), and *Amphiuma tridacylum* (three-toed amphiuma, n=2). Blood samples were collected antemortem via venipuncture of the ventral tail vein from *Cryptobranchus alleganiensis* (hellbender, n=5) and *Siren lacertina* (greater siren, n=6). Agarose gel electrophoresis was performed using plasma collected from each species. Electrophoretograms varied in the total number of fractions between each species; however, the number of fractions was consistent within a species. An albumin migrating fraction (predominant fraction in normal plasma samples of other species) was consistently observed in all species. Additionally, a pre-albumin migrating fraction was identified in species which primarily use other organs aside from the skin for respiration. These results provide a representative yet preliminary example of a normal plasma protein electrophoretogram for seven amphibian species. As electrophoretograms were observed to vary greatly among amphibian species, it is important for future studies to establish reference intervals and further classify protein fractions to fully evaluate protein electrophoresis as a useful tool in amphibian health investigations.

1. Introduction

Amphibians are currently facing severe threats to their biodiversity with at least 41% of amphibian species threatened with extinction (IUCN, 2020). With rates of amphibian declines increasing due to various factors, including emerging infectious disease, climate change, and habitat destruction (IUCN, 2020), it is now more important than ever to develop diagnostic tools to study amphibian health. One diagnostic tool which lacks appropriate investigation in amphibian species and can be used to assess health status is plasma protein electrophoresis.

Plasma protein electrophoresis is a routinely performed clinical test in numerous mammalian species, including humans (Tothova et al., 2016). This assay measures proteins in the blood as a means to characterize the health status of an individual, and in some cases, identify certain diseases such as inflammatory processes, lymphoproliferative processes, and abnormalities in multiple organ systems including the gastrointestinal tract, kidneys, and liver (Cray, 2021; Forzan et al., 2017). More recently, protein electrophoresis has become well-described in avian species and is becoming more commonly used in reptiles (Cray, 2021). Although the plasma proteins of amphibians have not been studied using modern electrophoresis tools and no reference intervals have

been calculated for any amphibian species, we can extrapolate data collected from other species to make generalizations about protein electrophoresis profiles in amphibians (Zaias & Cray, 2002).

Protein electrophoresis may be particularly useful in amphibians for two reasons. First, plasma protein electrophoresis can be performed on very small amounts of blood. Due to their frequently small size, blood volumes which can safely be obtained from live amphibians are often less than 0.5 ml depending on the species (Forzan et al., 2017). While this small amount of blood is limiting for many diagnostic tests, plasma protein electrophoresis offers a feasible diagnostic option in amphibians, including in antemortem settings. Second, protein electrophoresis provides a much more accurate measurement of plasma proteins than commonly used methods such as bromocresol green (BCG) methods (Cray, 2021). BCG methods, which are commonly used in mammalian species to quantitate albumin (the predominant fraction in normal plasma samples), have been shown to be inaccurate in non-mammalian species due to binding of BCG to albumin as well as globulins (Cray, 2021).

As in other non-mammalian species, plasma protein profiles in amphibians can vary greatly among species (Chen, 1967, 1970; Cray, 2021). They can also vary within species based on sex, age group/life stage, and potentially environmental factors (Chen, 1967, 1970; Cray, 2021; Forzan et al., 2017; Frieden et al., 1957; Herner & Frieden, 1960; Young et al., 2012). For these reasons, it is important that reference intervals are established for each species and that sex, age group/life stage, and environmental factors are considered. Reference intervals are useful to identify abnormalities in results of a diagnostic test. The goal of this brief report is to provide a representative example of a plasma protein electrophoretogram from a clinically normal individual or individuals from each of seven diverse amphibian species including one anuran and six urodelans. Future studies can build on these findings and strengthen our knowledge of plasma protein electrophoresis in amphibians.

2. Methods

Animals

Species examined included *Osteopilus septentrionalis* (Cuban tree frog, n=2), *Siren lacertina* (greater siren, n=6), *Gyrinophilus porphyriticus* (spring salamander, n=1), *Notophthalmus viridescens* (eastern newt, n=1), *Eurycea guttolineata* (three-lined salamander, n=2), *Amphiuma tridacylum* (three-toed amphiuma, n=2), and *Cryptobranchus alleganiensis* (hellbender, n=5). All individuals of each species aside from the hellbender were held in a human-managed setting and were experimental controls in chytridiomycosis trials. The hellbenders were collected in the wild from two separate sites in Tennessee (Middle and East) under Tennessee Wildlife Resources Agency (TWRA) Permit #1691. Cuban tree frogs and greater sirens were collected by Florida Fish and Wildlife Conservation Commission, the spring salamander was collected from North Carolina Wildlife Resources Commission, the eastern newt was collected from Tennessee under TWRA permit #1504, and three-toed amphiuma were collected from Louisiana under Louisiana Department of Wildlife and Fisheries permit #WDP-20-

077. Three-lined salamanders were captive bred and purchased from Indoor Ecosystems, LLC.

Animals (except hellbenders that were collected and sampled in the wild) were transported to and housed in the Johnson Animal Research and Teaching Unit at the University of Tennessee, Knoxville under either UTK-IACUC protocol #2723 (greater sirens) or UTK-IACUC protocol #2395 (all others). Greater sirens and three-toed amphiuma were individually housed in 3.78L tanks at room temperature (~20C). All other species were individually housed in separate containers within a Conviron environmental growth chamber. Individual housing for Cuban tree frogs, spring salamander, and three-lined salamanders consisted of a 710-cm^3 plastic container containing a moist paper towel and PVC cover object. Individual housing for the eastern newt consisted of a 2000-cm^3 plastic container that had 300-mL of water and a PVC cover object.

The spring salamander, three-lined salamanders, and three-toed amphiuma were skin swabbed for *Batrachochytrium dendrobatidis* (*Bd*) qPCR upon entry into the laboratory and confirmed to be negative. Cuban tree frogs, eastern newts, and sirens were heat-treated for *Bd* infection upon arrival into the laboratory without initial *Bd* qPCR testing. Heat treatment consisted of 10 days in an environmental growth chamber (Conviron, Winnipeg, Canada) at 30°C. Animals were then skin swabbed and confirmed to be negative for *Bd* using qPCR.

Following heat treatment, individuals were acclimated over 2 weeks to a temperature of 14°C. Environmental chambers were also set to a light/dark cycle that corresponded with the season associated with this temperature (spring/fall = 14 hours dark). Water changes were performed once every three days for individuals housed in environmental growth chambers. All human-managed individuals were fed 4% of their body mass 24 hours prior to every water change. The eastern newt and greater sirens were fed bloodworms (*Chironomus plumosus*), three-toed amphiuma were fed earthworms and bloodworms, Cuban tree frogs and spring salamander were fed crickets, and the three-lined salamanders were fed fruit flies.

All animals (except hellbenders) were control individuals in chytridiomycosis studies; therefore, they were inoculated with autoclaved, dechlorinated water for 24 hours in substitution of a chytrid zoospore dose at the beginning of the experimental trial. Animals were skin swabbed once every 6 days following the previously described methods (10 swabs along the ventrum and 5 along the bottom of each foot). Health checks consisting of brief visual examination were performed twice daily. All species were euthanized within 90 days post-entry into the laboratory.

Blood/Sample Collection

Blood was collected from hellbenders in the field. Hellbenders were restrained using a towel wrap soaked in river water with only the tail exposed. Blood was collected from the ventral tail vein using a heparinized syringe. A skin swab was collected at the time of blood collection and processed for *Bd* qPCR using methods similar to Boyle et al (2004). All animals were confirmed to be qPCR negative for *Bd*.

Blood was collected from sirens while under anesthesia. Anesthesia was induced by water bath exposure to 200 mg/L benzocaine dissolved in dechlorinated water. During blood collection, each siren was wrapped in a towel soaked in the water/benzocaine solution, its tail exposed and 1.5–2 ml of blood was collected from the ventral tail vein using a heparinized syringe. Blood samples for the remaining species were collected immediately postmortem through cardiocentesis using a heparinized capillary tube.

Blood Processing and Analysis

After collection, blood from all species was spun in an Eppendorf 5424R centrifuge at 2170 G for 3 minutes to separate plasma. Samples were analyzed in accordance with instructions provided by the Helena QuickGel system with the use of Split Beta gels (Helena Laboratories, Inc., Beaumont, Texas 77707, USA). Samples were run in duplicate. A known human control sample (Helena Laboratories, Inc.) as well as a known abnormal canine sample from a canine known to have multiple myeloma were also run to confirm accuracy. Gels were scanned and analyzed using software provided by Helena.

Statistical Analysis

To compare the variation in fraction sizes between species to the variation in fraction sizes within a species, the compositional mean of the albumin fraction as well as the fraction immediately cathodic to albumin was calculated for the species that had samples from more than one individual. The metric variance was then determined between all species and within species for the hellbenders and the sirens. All analyses were performed using the 'compositions' package in R (version 4.1.1) (Boogart & Tolosana-Delgado, 2013).

3. Results

Plasma protein fractions from each species aside from the hellbenders are presented in the appendix in Table 3.1. Plasma protein fractions along with site of collection for hellbenders are presented in Table 3.2. Representative plasma electrophoretograms from each amphibian species along with human control and canine with multiple myeloma are presented in Figure 3.1. Plasma protein profiles varied greatly in the number of protein fractions among amphibian species, with 9 fractions observed in the Cuban tree frog and 3 in the greater siren.

All amphibian samples examined had what is suspected to be an albumin migrating fraction, which represented the largest fraction in all species aside from the hellbender. The largest fraction in the majority of hellbenders was the fraction migrating to the cathode. Four species (Cuban tree frogs, three-toed amphiuma, eastern newt, and greater sirens) had a pre-albumin migrating fraction anodic to the albumin fraction. Hellbenders, three-lined salamanders, and spring salamanders lacked this fraction. Number and size of fractions cathodic to the albumin fraction varied among species. The metric variance of the albumin fraction and the fraction cathodic to albumin between all species was 0.44, the metric variance of these fractions between hellbenders was 0.13, and the metric variance of these fractions between sirens was 0.08, indicating that between-species variability in these fractions was larger than within-species variability.

4. Discussion

This study provides preliminary representative electrophoretograms of a clinically normal individual of seven amphibian species. No plasma protein electrophoretograms using modern automated gel technology have been previously published for any of these species. The sample sizes used in this study were small, and therefore, reference intervals were not able to be determined (Friedrichs et al., 2012). Therefore, this work supplies basic groundwork for future studies to build on this information and provide useful reference intervals for these species.

Marked variation in plasma protein electrophoretograms between amphibian species, even within the same order was identified in study. Fraction number and size varied among each of the species examined, and fraction size varied within members of the same species. However, variation in fraction sizes between species was determined to be greater than variation in fraction sizes within a species. Previous studies have shown that electrophoretograms vary greatly between amphibian species, and even within a species, protein fractions can vary between life stages. Studies using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) have identified between 11 and 20 protein fractions in amphibian serum (Chen, 1967, 1970). More recent studies using either cellulose acetate electrophoresis (CAE) or AGE identified either 4 or 5 protein fractions, respectively, in two anuran species (Chiesa et al., 2006; Young et al., 2014). Additionally, multiple studies using paper electrophoresis or SDS-PAGE have discovered that protein fractions change throughout metamorphosis in anuran species (Chen, 1970; Frieden et al., 1957; Herner & Frieden, 1960). This highlights the importance of evaluating every amphibian species individually and creating reference intervals for each.

In agarose gel electrophoresis (AGE), plasma proteins separate into fractions including albumin and three types of globulins (alpha, beta, and gamma) (Cray, 2021). Albumin is the predominant fraction in normal plasma samples. Some non-mammalian species also have an additional fraction anodic to the albumin fraction which is conventionally referred to as a pre-albumin fraction (Cray, 2021). Four of the seven amphibian species in this study (Cuban tree frog, three-toed amphiuma, eastern newt, and greater siren) had a pre-albumin migrating fraction. Interestingly, each of the species with a pre-albumin migrating fraction primarily rely on organs other than their skin (external gills, lungs, or a combination of both) for respiration (Wells, 2007). The species that lack this fraction (hellbender, three-lined salamander, and spring salamander) primarily rely on their skin for respiration along with varying but lesser degrees of buccopharyngeal membrane respiration (Wells, 2007). The significance of this finding is uncertain. However, as prealbumin has been associated with binding thyroxine and retinol in avian species, this result might suggest a difference in one or both of these parameters among the two groups, and perhaps is linked to respiration (Lumeji, 2008). An important next step to elucidate the biological significance of the pre-albumin migrating factor will be to assess whether this pattern holds across larger sample sizes and use mass spectrometry to confirm identification of proteins within each fraction.

Protein electrophoretograms of all species appeared to have what would be an albumin fraction based on the migration characteristics of well-documented species

(Cray, 2021). Previous studies using various methods of electrophoresis, as well as staining reaction techniques have also suggested that amphibian species have one to two albumin migrating peaks, which can vary in size (Chen, 1967, 1970; Chiesa et al., 2006; Cray, 2021; Francis et al., 1985; Frieden et al., 1957; Herner & Frieden, 1960; Young et al., 2012; Young et al., 2014). This is an important finding as changes in albumin levels can be useful in diagnosing many pathologic processes including active inflammation, dehydration, liver, gastrointestinal, or renal disease, as well as malnutrition (Forzan et al., 2017). While its utility in amphibians is not fully understood, in Chapter I, albumin levels were associated with *Batrachochytrium salamandrivorans* (*Bsal*) infection status in *Taricha granulosa*. Individuals which were clinically diseased with *Bsal* chytridiomycosis had increased levels of albumin due to dehydration. If dehydration can be identified early, then appropriate therapeutic options such as fluid therapy can be pursued in captive settings to mitigate the effects of *Bsal* chytridiomycosis.

In addition to the fraction suspected to represent albumin, two to eight additional fractions were identified in this study depending on the species. Past amphibian studies have labeled the fractions cathodic to albumin as globulins in accordance with mammalian electrophoretograms (Chiesa et al., 2006; Young et al., 2014). One study used various staining reaction methods to identify these proteins as transferrin, haptoglobin, lipoprotein, and glycoprotein (Chen, 1970). Monitoring of globulin levels is useful for many reasons including evaluation of the immune response (Cray, 2021). A study involving infection with a different chytrid species, *Batrachochytrium dendrobatidis* (*Bd*), showed failure of protein fractions 2 and 5 (labeled by the author as alpha-1 globulins and gamma globulins, respectively) to increase in response to immune stimulation in animals infected with *Bd* chytridiomycosis compared to controls (Young et al., 2014). If these fractions do represent globulins, these findings potentially indicate pathogen-induced immunosuppression, which is an aspect of disease pathogenesis previously associated with *Bd* chytridiomycosis (Young et al., 2014).

It is important to note that in order for protein fractions to be definitively classified in amphibians, further investigations including mass spectrometry must be performed. Special staining reactions used in previous studies to aid in protein fraction classification are not entirely specific. Additionally, it is not accurate to assume protein fractions in amphibians have identical compositions to those described in other species, although they likely have similar properties. Mass spectrometry could further classify what each protein fraction represents by assessing the weight and charge of all proteins present in each fraction. These results could then be compared to what is known in other species such as mammals.

Limitations of this study include small sample size and lack of supporting biochemical and hematologic analysis. Supportive blood profiles are useful for interpreting the significance of plasma protein electrophoresis values. Furthermore, including multiple animals of different sexes, development stages, and geographic locations over multiple seasons will enable development of more appropriate reference intervals. Lastly, classification of the protein fractions by mass spectrometry is necessary and may explain variations among species who primarily utilize their skin as a respiratory organ versus those that incorporate other means of respiration.

Overall, plasma protein electrophoresis is an important tool for detecting many pathologic conditions including inflammation as well as monoclonal and polyclonal gammopathies (Cray, 2021). Based on previously mentioned trends in alterations of fraction levels in diseased animals versus controls, plasma protein electrophoresis could prove to be a useful tool in helping to identify severity of disease in amphibians (Chapter I). It could also be useful to guide treatment protocols and explore the pathogenesis of infectious or non-infectious diseases. Furthermore, plasma protein electrophoresis requires only a very small amount of blood which allows it to be performed antemortem in many amphibian species and has the potential to be used at the population level for disease surveillance purposes.

An additional potential use for plasma protein electrophoresis is routine health monitoring for amphibians in research, head-start, or other human-managed facilities. As many amphibian populations are declining due to emerging infectious disease, climate change, and habitat destruction (IUCN, 2020), many species are being brought into repatriation programs in attempt to save them from extinction. In these situations, every animal is significant and therefore health status must be closely monitored. By combining plasma protein electrophoresis with other clinical pathology measurements, a more complete overview of health may be obtained.

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APPENDICES

Appendix A: Tables

Table 3.1 Protein fraction results for each clinically normal individual amphibian.

			Percentage out of 100								
Species	**Animal ID**	**Composite Photo (Y/N)**	**Fraction 1**	**Fraction 2**	**Fraction 3**	**Fraction 4**	**Fraction 5**	**Fraction 6**	**Fraction 7**	**Fraction 8**	**Fraction 9**
Osteopilus septentrionalis	CTF1	Y	4.68	28.91	17.91	18.77	11.59	6.71	4.17	3.18	5.07
Osteopilus septentrionalis	CTF2	N	1.48	34.67	15.96	19.01	11.94	4.97	4.29	1.97	5.7
Siren lacertina	SL1	N	22.15	45.85	31.99	-	-	-	-	-	-
Siren lacertina	SL3	Y	19.13	59.25	21.62	-	-	-	-	-	-
Siren lacertina	SL4	N	29.15	44.94	25.91	-	-	-	-	-	-
Siren lacertina	SL5	N	23.43	50.94	25.63	-	-	-	-	-	-
Siren lacertina	SL6	N	19.07	58.85	22.08	-	-	-	-	-	-
Siren lacertina	SL8	N	30.78	41.95	27.28	-	-	-	-	-	-
Gyrinophilus porphyriticus	GY1	Y	32.62	30.81	8.58	13.55	8.63	5.8	-	-	-
Notophthalmus viridescens	D2	Y	19.93	32.99	13.23	28.28	5.57	-	-	-	-
Eurycea guttolineata	EG4	N	64.73	12.07	19.23	3.97	-	-	-	-	-
Eurycea guttolineata	EG5	Y	61.02	12.75	22.45	3.78	-	-	-	-	-
Amphiuma tridacylum	AT1	Y	4.26	34.50	6.15	19.54	14.77	20.77	-	-	-
Amphiuma tridacylum	AT2	N	5.22	31.64	8.42	19.43	19.25	16.04	-	-	-

Table 3.2 Protein fraction results for individual *Crytobranchus alleganiensis* (hellbenders).

		Percentage out of 100			
Animal ID	**Location**	**Fraction 1**	**Fraction 2**	**Fraction 3**	**Fraction 4**
TN1	East TN	31.07	15.92	14.26	38.75
TN2	Middle TN	35.73	10.3	19.93	34.04
TN3	East TN	22.51	17.13	24.66	35.70
TN4	East TN	19.96	16.21	20.28	43.56
TN5	East TN	36.49	13.63	20.50	29.38

Appendix B: Figures

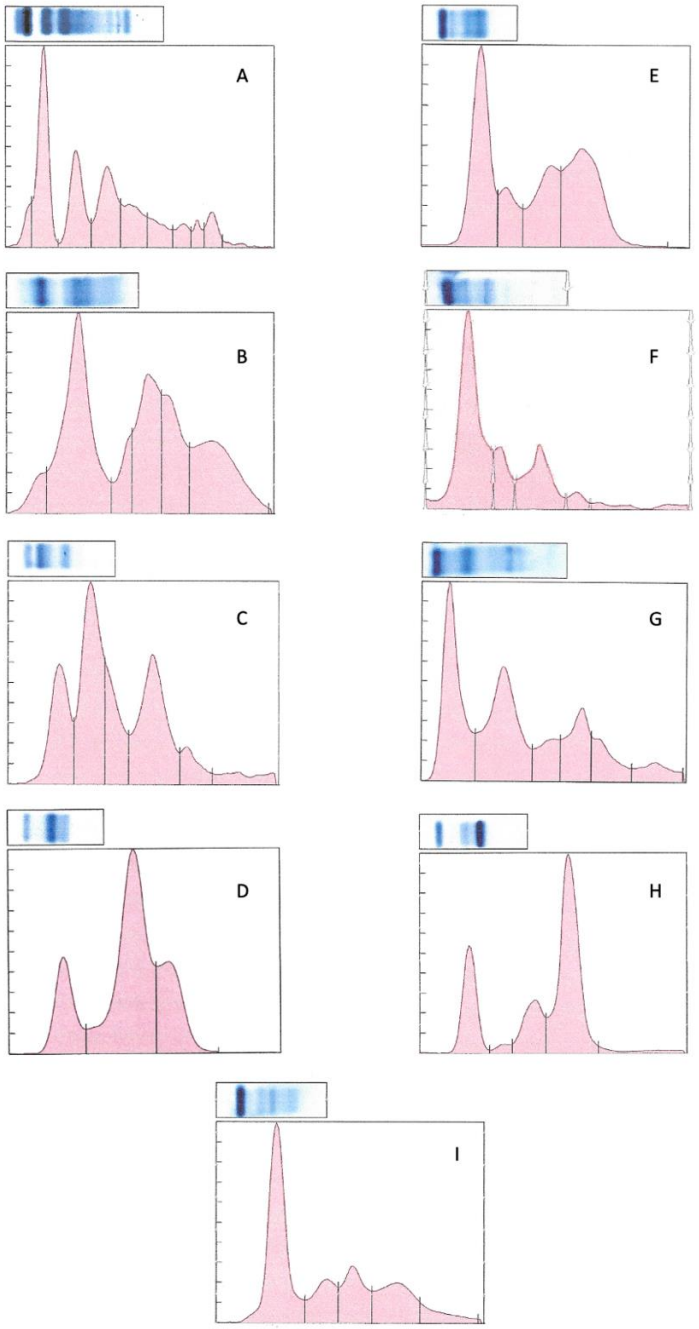

Figure 3.1 Representative agarose gel electrophoresis (AGE) electrophoretograms of heparinized plasma from a Cuban tree frog (A), three-toed amphiuma (B), eastern newt (C), greater siren (D), hellbender (E), a three-lined salamander (F), a spring salamander (G), serum from a canine with multiple myeloma (H), and serum from a human control (I). The y-axis represents the size of each protein fraction, and the x-axis represents the migration distance of each protein fraction from the anode (left) to cathode (right).

CONCLUSION

As amphibian species continue to suffer from devastating declines, disease investigations and health monitoring are now more important than ever. The emerging fungal pathogen *Bsal*, is causing chytridiomycosis and disease-induced declines in salamander species in Europe and poses a significant threat to North American salamander biodiversity (Basanta et al., 2019; Carter et al., 2021; Grant et al., 2017; Lotters et al., 2020; Richgels et al., 2016; Yap et al., 2015; Yap et al., 2017). However, we know little about the mechanisms of its pathogenesis and disease progression, particularly for North American salamanders. Identifying how, why, and under what conditions *Bsal* causes disease is a critical foundation for basic disease ecology, more efficient disease surveillance, and the parameterization of predictive models of *Bsal* spread and population-level impacts. Additionally, investigation into the use of supplementary diagnostic tools for health and disease monitoring in these species is warranted. I addressed the pathogenesis of *Batrachochytrium salamandrivorans* (*Bsal*) and compared it to what is known about the related pathogen, *Batrachochytrium dendrobatidis* (*Bd*), investigated the effect of environmental temperature and zoospore exposure dose on *Bsal* infection and disease development, and have set out baseline plasma protein electrophoretograms for clinically normal individuals of one anuran and six urodelan species.

I found that electrolyte imbalances and dehydration secondary to damage to the epidermal cells by *Bsal*-induced skin lesions are likely the primary factors in disease pathogenesis. Additionally, a systemic immune response is incited in clinically diseased *T. granulosa* which may be either primarily related to *Bsal* infection or could be associated with secondary bacterial infection. Future studies should further investigate the role that secondary bacterial infection plays in this disease.

I also found that environmental temperature and *Bsal* zoospore dose each have a significant effect on disease progression and confirmed that 14°C is the ideal temperature for *Bsal* to infect its hosts and incite damage to the epidermis. *Notophothalmus viridesens* exposed at 22°C did not become infected, and *N. viridescens* exposed at 6°C developed fewer lesions and had longer survival times than those exposed at 14°C. The hindlimbs, cloacal region, and tail had on average the highest number of and most severe skin lesions. This information is important for disease surveillance efforts as it provides knowledge regarding the best anatomic locations to examine and collect diagnostic samples from to have the highest chance of detecting the pathogen in *Bsal*-infected animals.

Overall, these studies elucidate the pathogenesis of *Bsal* chytridiomycosis and describe how environmental temperature affects disease progression. Additionally, findings provide support for the use of potential disease mitigation strategies against *Bsal* chytridiomycosis in captive settings. By knowing that the pathogenesis involves electrolyte imbalances and dehydration, fluid therapy including supplemental electrolytes could be used in disease treatment. Additionally, the incorporation of antibiotics may be useful as the systemic inflammatory response I identified in clinically diseased *T.*

granulosa may have been at least in part due to secondary bacterial infection. Heat treatment is also a viable treatment option in *N. viridescens*.

In regard to the representative plasma protein electrophoretograms from clinically normal individuals of various amphibian species, these can be used as a baseline example for future studies. These investigations should involve the development of reference intervals in multiple species and across different sexes, environmental conditions, and disease states. This information will aid in the understanding of disease processes, as well as monitor health status, especially in critically endangered species.

Through combining multiple diagnostic modalities including both clinical and anatomic pathology tools, I have strengthened our understanding of amphibian health as well as the pathogenesis of an important emerging infectious disease in these species. Future studies can use this work as an example of the advantages to this multimodal approach. By utilizing techniques that investigate different aspects of amphibian health, we can work towards improving our conservation efforts for these important and declining species.

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