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Environmental temperature and pathogen dose affect histologic lesion count and severity in *Notophthalmus viridescens* infected with *Batrachochytrium salamandrivorans*

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Introduction: *Batrachochytrium salamandrivorans* (*Bsal*) was discovered a decade ago in Europe, where it is emerging and decimating salamander populations. North America, a global hotspot for salamander biodiversity, faces risk of Bsal introduction through trade or other pathways. An abundant salamander species in these systems, the eastern newt *Notophthalmus viridescens*, is highly susceptible to Bsal and may play an important role in Bsal epidemiology if the pathogen is introduced. However, we know very little about the physiological mechanisms contributing to the pathogenesis of *Bsal* chytridiomycosis. This limits our ability to treat infection on an individual level and predict the evolutionary responses of resistance and tolerance on the population level following Bsal invasion.

Methods: We tested the hypothesis that morbidity and mortality of *Bsal*-infected individuals are directly related to skin lesions, after controlling for Bsal infection intensity. To test this, we compared *Bsal*-induced lesions in eastern newts among four *Bsal* zoospore doses (5x103–6 per 10 mL) and maintained at three environmental temperatures (6, 14, and 22°C). Following euthanasia, animals were processed for histologic examination and *Bsal*-associated lesions were counted and graded for severity on a scale of 1–5. Additionally, dermal glands were examined for *Bsal* invasion and all internal organs were assessed.

Results: Newts exposed at 22°C did not become infected by *Bsal*. Newts exposed at 14°C had more lesions compared to those exposed at 6°C across all zoospore doses. For the lowest three zoospore exposure doses, as zoospore dose increased, so did lesion count. Additionally, there was a strong negative relationship between lesion count and survival, after accounting for Bsal infection intensity, suggesting that lesions are contributing to *Bsal* pathogenesis beyond infection intensity. Lesions were most abundant in the hindlimbs, cloacal region, and tail. There were no Bsal-related abnormalities in internal organs; further supporting our hypothesis that morbidity and mortality in infected individuals are directly related to skin lesions.

Discussion: This is the first Bsal susceptibility study which has evaluated the number, distribution, and severity of histologic lesions in *Bsal*-infected hosts

across multiple temperatures. 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 locally increased lesion prevalence.

KEYWORDS

Bsal, chytridiomycosis, histopathology, newt, salamander

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 the cause of the majority of these declines (Berger et al., 1999; Yap et al., 2015). *Bsal*, which was discovered more recently in 2013, is more pathogenic to salamanders, and is emerging quickly (Scheele et al., 2019). *Bsal* is presumed to have originated in Asia and spread to Europe through trade of infected animals (Martel et al., 2014; Yap et al., 2017), where it is currently causing large die-offs in multiple salamander species (Martel et al., 2014; Lotters et al., 2020; 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 in the wild, species susceptibility, and presence of *Bsal* in trade, it is likely only a matter of time before *Bsal* spreads to regions other than Asia and Europe (Gray et al., 2015; Grant et al., 2017; Yap et al., 2017; Carter et al., 2019; Carter et al., 2021; Gray et al., 2023). 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 host resistance and tolerance to this emerging pathogen (Raberg et al., 2009). It also can provide insight into the likelihood of infection being maintained and disease developing in captive amphibian trade.

Environmental temperature is especially important for amphibians as many physiologic functions, including the immune response, can be significantly affected by changes in temperature (Wells, 2007; Rollins-Smith, 2017; Rollins-Smith, 2020). Previous studies have shown the pathogenicity of *Bsal* can change with changing temperatures in both eastern newts as well as fire salamanders (*Salamandra salamandra*) (Blooi et al., 2015a; Stegen et al., 2017; Beukema et al., 2021; Carter et al., 2021). Carter et al (Carter et al., 2021). 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. 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 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 (Martel et al., 2014; Wilber et al., 2021; Gray et al., 2023). 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 for multiple urodelan species, number of *Bsal*-induced skin lesions visible grossly is not predictive of survival and only has a weak positive correlation with *Bsal* qPCR load (Wilber et al., 2021). This may be because lesions are being missed without the use of histology. 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 better overall picture of disease progression in an 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 exposure doses on survival rate and determined that this pathogen causes dose-dependent mortality in multiple species (Carter et al., 2019; Carter et al., 2021). Utilizing preserved specimens from Carter et al (Carter et al., 2021), which were exposed to varying *Bsal* doses at three temperatures, we analyzed how disease induced mortality rate and *Bsal* infection load relate to histological lesion counts and grades. Overall, the aim of this study was to better understand how *Bsal* causes morbidity in susceptible amphibian species.

2 Methods

2.1 Ethics statement

Husbandry as well as euthanasia procedures are described in detail in Carter et al (Carter et al., 2021), and 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). *Notophthalmus viridescens* that reached euthanasia endpoints were humanely euthanized via transdermal exposure to benzocaine hydrochloride.

2.2 Animals

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

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 (Chatfield and Richards-Zawacki, 2011; Bletz, 2013). 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 a previously described protocol (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.

2.3 Experimental design

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.

2.4 Experimental infection

Bsal was isolated from a fire salamander (*Salamandra salamandra*) in the Netherlands (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 (Carter et al., 2019).

Experimental infection was performed according to previously described methods by Carter, et al (Carter et al., 2021). *Bsal*-exposed individuals were exposed to either a dose of $5x10^3$, $5x10^4$, $5x10^5$, or $5x10^6$ zoospores per 10 mL. This was done by separately placing each individual into a 100-mL plastic tube which contained a mixture of *Bsal* zoospores and 9-mL of autoclaved dechlorinated water for 24 hours in the environmental incubators. Control animals were placed into similar tubes containing 10-mL of autoclaved dechlorinated water under identical time and environmental conditions as the exposed animals.

2.5 Histopathology

Methods for processing carcasses for histopathologic assessment were similar to those used by Sheley, et al., 2023 (Sheley et al., 2022). Carcasses were stored in 10% neutral buffered formalin for at least 48 hours prior to processing. Transverse sections through the body were taken at approximately 3-mm intervals and legs were removed at either the scapulohumeral or coxofemoral joint. Ten standardized sections (Table 1 & Figure 1) were placed into tissue cassettes. Cassettes containing tissues were decalcified for 24 hours using a solution containing formic acid. Tissues were then processed and stained routinely with hematoxylin and eosin (H&E). Histologic examination consisted of assessing 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

TABLE 1 Anatomic location associated with each of ten standardized sections examined histologically for lesion counts and grading in *Batrachochytrium salamandrivorans* infected *N. 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

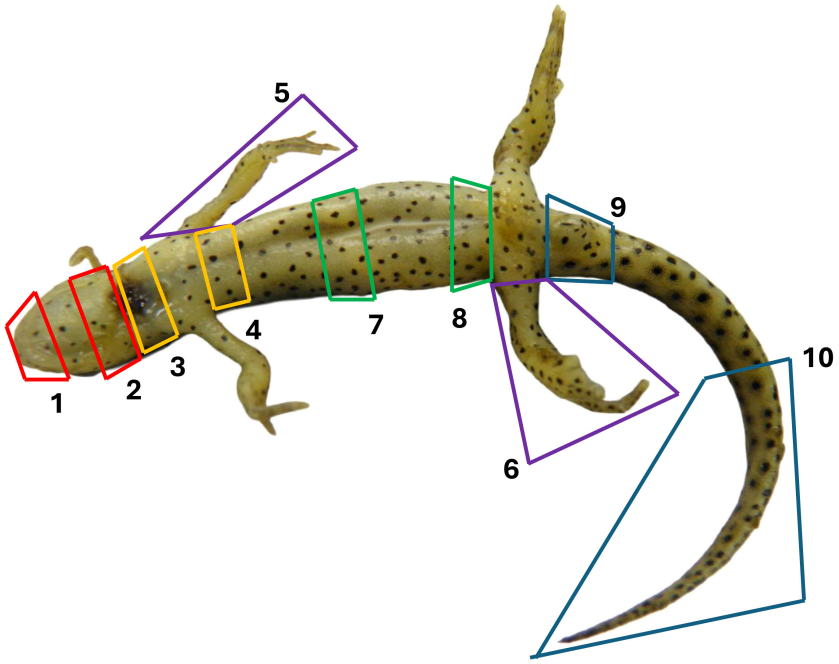

FIGURE 1
Anatomic location associated with each of the ten standardized sections examined histologically for lesion counts and grading in *Batrachochytrium salamandrivorans* infected *N. viridescens*.

membrane which were ≥1 millimeter long; Table 2; Figure 2). Excelis Accu-Scope software was used to measure the perimeter of each section to standardize measures of lesion counts to counts per unit area of cross section.

2.6 Quantitative polymerase chain reaction

At the time of necropsy, animals were skin swabbed following the standardized protocol previously mentioned of 10 swipes along the ventrum and 5 swipes along the plantar surface of each foot. To detect *Bsal* and estimate loads, genomic DNA was extracted from each skin swab using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and qPCR performed similar to previously described methods (Blooi et al., 2015a) using the Applied Biosystems Quantstudio 6 Flex qPCR instrument (Thermo Fisher Scientific Inc). Samples were all run in duplicate and confirmed positive if both replicates reached cycle threshold prior to 50 amplification cycles (Carter et al., 2019).

TABLE 2 Description of histologic lesion grading system used in *Batrachochytrium salamandrivorans* infected *N. 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

2.7 Statistical analysis

2.7.1 Lesion counts

To determine how temperature and *Bsal* zoospore exposure dose affected the number of lesions per histologic cross section, a negative binomial generalized linear model was fit where the response variable was lesion count per cross section perimeter and the predictor variables were temperature, *Bsal* zoospore exposure dose, an interaction term between temperature and *Bsal* zoospore exposure dose, and section number. Environmental temperature included 6°C and 14°C, *Bsal* zoospore exposure dose included four levels $5x10^{3-6}$, and section number included sections 1–10, corresponding to various anatomical sites of the animal's body (Table 1; Figure 1). Initial analyses showed that lesion counts were over-dispersed relative to a Poisson distribution. Therefore, a negative binomial distribution was used to describe lesions. Additionally, lesion counts were obtained from histological cross sections with varying perimeters. This meant that a larger perimeter might 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, a log (total perimeter) offset term was

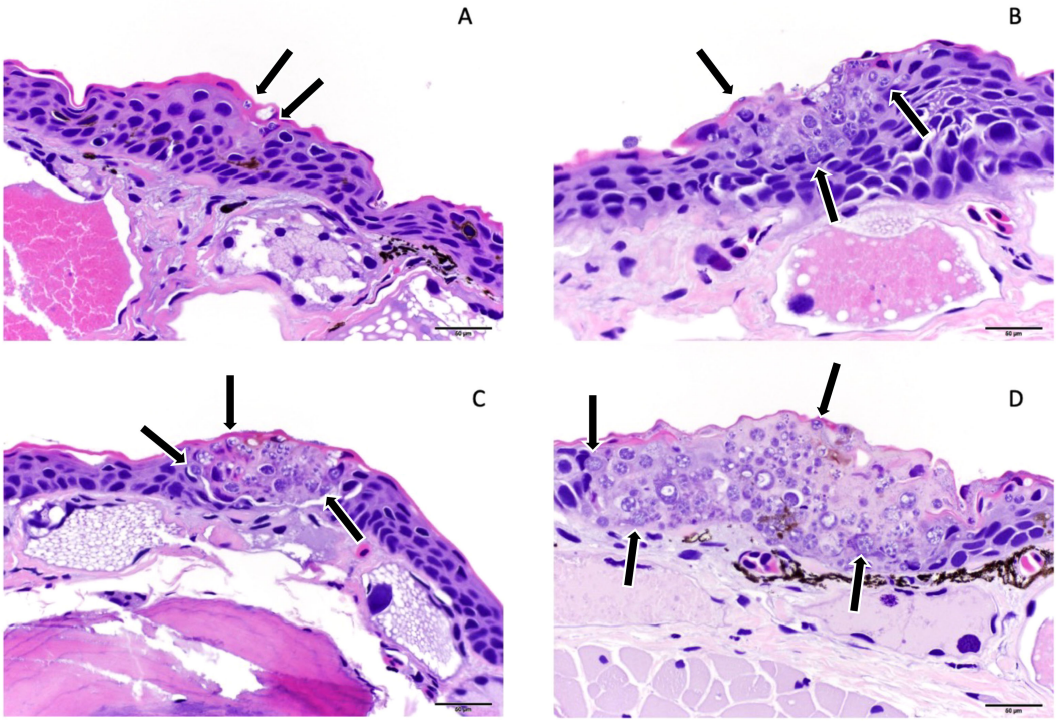

FIGURE 2
Examples of histologic lesion grading scheme used for *N. viridescens* infected with *Batrachochytrium salamandrivorans* (*Bsal*) including Grade 1 **(A)**, Grade 2 **(B)**, Grade 4 **(C)**, and Grade 5 **(D)**. Grade 1 lesions were limited to *Bsal* organisms (black arrows) within the stratum corneum, grade 2 lesions included *Bsal* organisms (black arrows) and associated cellular damage which extended into the mid-epidermis, grade 4 = coalescing/large lesions with *Bsal* organisms (black arrows) and associated cellular damage which extended through the epidermis to the basement membrane which were <1 millimeter in length, grade 5 lesions included coalescing/large lesions with *Bsal* organisms (black arrows) and associated cellular damage which extended full thickness through the epidermis and were ≥1 millimeter in length.

included in the negative binomial regression (Faraway et al., 2016). The lesion count model was fit using the brms package in R (Burkner, 2017), with a weakly regularizing prior on the effect sizes for the predictor variables temperature, *Bsal* zoospore exposure dose, and section number. Convergence was ensured when fitting the models by visually examining chains and checking that R_hat (the potential scale reduction factor) < 1.01 and effective sample size > 400 for all parameters (Vehtari et al., 2020). Five candidate models were fit to the data and best predictive model was selected using Pareto smoothed importance sampling leave-one-out information (PSIS-LOO (Vehtari et al., 2016),). The lower the PSIS-LOO of the model, the better the predictive performance. The models we compared to the final model described above included the following combinations of predictors: 1) temperature and *Bsal* zoospore exposure dose, 2) temperature, *Bsal* zoospore exposure dose, and temperature and *Bsal* zoospore exposure dose as an interaction term, and 3) temperature, *Bsal* zoospore exposure dose, and section number.

2.7.2 Probability of lesion presence

To determine how temperature and *Bsal* zoospore exposure dose affected the probability of lesion presence, a Bernoulli generalized linear model was fit where the response variable was lesion presence (1 = lesions present, 0 = lesions absent) and the predictor variables were *Bsal* zoospore exposure dose, temperature, an interaction term between *Bsal* zoospore exposure dose and temperature, and the scaled log (perimeter) (where we scaled by subtracting the mean and dividing by the standard deviation) 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. The model was fit using the brms package in R (Burkner, 2017), with a weakly regularizing prior on the effect sizes for the predictor variables temperature, *Bsal* zoospore exposure dose, and perimeter. When fitting the models, model convergence was ensured using the previously described methods. Four candidate models were fit to the data and the best predictive model was selected using PSIS-LOO. Models compared to the final model described above included the following combinations of predictors: 1) the scaled log(perimeter) (where we scaled by subtracting the mean and dividing by the standard deviation), 2) *Bsal* zoospore exposure dose, temperature, and scaled log (perimeter), 3) *Bsal* zoospore exposure dose, temperature, *Bsal* zoospore exposure dose and temperature as an interaction term, scaled log(perimeter), and section number.

2.7.3 Lesion grades

To determine how temperature and *Bsal* zoospore exposure dose affected the expected lesion density of each lesion grade per unit cross section, a generalized linear model was fit 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 exposure 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 et al., 2016). Originally, lesion grades 1–5 were included in the model; however, grade 5 lesions were dropped from the analysis due to their relative rarity leading to issues with separability. Models were fit 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, an offset term of log(total perimeter) was included in the log-linear regression as described in the lesion count analysis.

The model was fit using the brms package in R (Burkner, 2017), with a weakly regularizing prior on the effect sizes for the predictor variables temperature, *Bsal* zoospore exposure dose, and lesion grade. All continuous covariates were standardized to a mean of 0 and a standard deviation of 1 before fitting. When fitting the models, model convergence was ensured using the previously described methods. Eight candidate models were fit to the data and the best predictive model was selected using PSIS-LOO. Models compared to the final model described above included the following combinations of predictors (using both a negative binomial distribution and a Poisson distribution for each): 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 exposure 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 exposure dose as an interaction term, the combination of lesion grade, temperature, and *Bsal* zoospore exposure dose as an interaction term, and individual ID as a random effect. For potentially influential observations with a Pareto smoothing k > 0.7, these values were temporarily excluded to see if they affected model selection. Model selection was unaffected by these potentially influential observations, and they were included in the model inference. Finally, *Bsal* zoospore exposure dose was replaced with *Bsal* qPCR load at the time of necropsy in all models and were compared to original models using PSIS-LOO.

2.7.4 Survival analysis

To determine how *Bsal* zoospore exposure dose and lesion count affected survival rate, a Cox proportional hazards model was fit where the response variable was days survived and the predictor variables were *Bsal* zoospore exposure dose and lesion count. We standardized as lesion counts using the following approach. Lesion counts and perimeters were added together across all cross sections (sections 1 – 10) and total lesion count was divided by total perimeter for each individual, yielding the quantity total lesion count per unit length of cross section. This quantity was then multiplied by the average perimeter of a cross section. This allowed us to interpret the resulting coefficient from the survival model as the change in log hazard given an increase of one lesion in the average cross section. Cox proportional hazards models were also used in Carter et al (Carter et al., 2021). However, they only examined the relationship of survival to exposure dose. We were specifically interested in whether lesion count explained additional variability in survival after accounting for *Bsal* load. We fit two models i) survival ~ *Bsal* load at death/censoring + standardized_lesion_count and ii) survival ~ (*Bsal* load at death/censoring + standardized_lesion)*temperature. The second model allowed us to test whether temperature affected the effect of lesion count or *Bsal* load on host survival. If *N. viridescens* were euthanized after reaching humane endpoints, they were designated as right-censored in the analysis.

When fitting the models, it was ensured that the proportional hazards assumption was met by confirming a non-significant relationship between Schoenfeld residuals for each covariate and time (Klein and Moeschberger, 2003). Overall goodness-of-fit was checked by plotting cumulative hazard against Cox-Snell residuals and visually assessing for clear outliers by plotting deviance residuals against observation number. All models were fit with using the coxph function in the `survival` package in R [version 4.1.1 (Therneau and Lumley, 2015)].

3 Results

3.1 Lesion counts

Across all *Bsal* zoospore exposure doses, *N. viridescens* exposed at 14°C had a higher lesion count per average cross section than those exposed at 6°C (Figure 3), though the effect of temperature changed with zoospore exposure dose (Difference in PSIS-LOO for models with and without the temperature by zoospore exposure dose interaction = 41.2; Figure 3). *N. viridescens* exposed to the $5x10^5$ zoospore dose at 14 °C had the overall highest lesion count per average cross section (number of lesions per average cross section for the $5x10^5$/14°C treatment: Mean (E): 77.24, 95% Credible Interval (CI): 55.70–109.15; Figure 3). Due to the significant interaction between temperature and zoospore exposure dose, individuals exposed to the $5x10^6$ zoospore dose at 14°C had lower lesion counts than those at $5x10^5$ doses (Difference between $5x10^5$ and $5x10^6$ at 14°C: E: 41.92, 95%CI: 22.31–69.78; Figure 3). Across all zoospore exposure dose and temperature combinations, sections 6 (hindlimb), 9 (cloacal region) and 10 (tail) had the highest lesion counts relative to the baseline of Section 1 (effect sizes relative to Section 1: [(section 6 = E: 0.57;

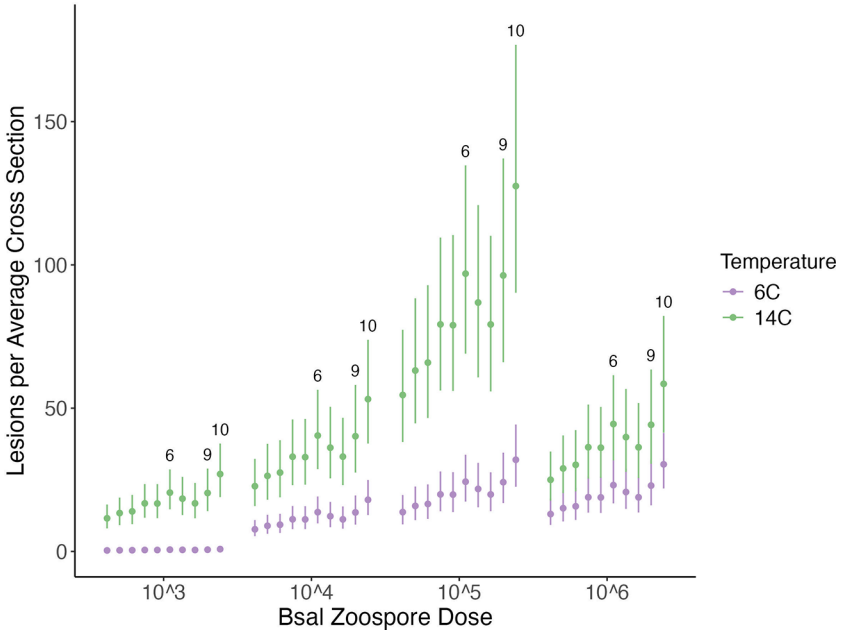

FIGURE 3
Plot of the histologic lesions per average cross section (y-axis) for each *Batrachochytrium salamandrivorans* (*Bsal*) zoospore exposure dose (x-axis) and temperature (6 °C = purple; 14 °C = green) treatment combination in *Bsal*-infected *N. viridescens*. Each point within a zoospore exposure dose represents a section in numerical order from section 1–section 10. Error bars are showing the 95% credible intervals around the predicted response.

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)]; Figure 3).

3.2 Probability of lesion presence

N. 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 $5x10^3$: 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 $5x10^4$: E: 0.15, 95%CI: 0.07–0.27; Figure 4). *N. 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 $5x10^5$: -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 $5x10^6$: -0.02–0.02; Figure 4). Finally, *N. viridescens* exposed to zoospore doses $5x10^{4–6}$ were more likely to have lesions present than those exposed to a zoospore dose of $5x10^3$ regardless of the temperature (Figure 4).

3.3 Lesion grade

Regardless of temperature, Grade 1 lesions were the most common at the lowest zoospore exposure dose, Grade 2 lesions were the most common at the $5x10^5$ zoospore exposure dose, and Grade 3 lesions were the most common at the $5x10^4$ and $5x10^6$ zoospore exposure doses (Figure 5). Grade 5 lesions were the least common grade of lesion across all zoospore exposure dose/temperature combinations (Total number of Grade 5 lesions observed from *N. viridescens* across all zoospore exposure dose/temperature combinations = 83). Grade 4 lesions were the second least common grade of lesions across all zoospore exposure dose/temperature combinations (Figure 5). Similar to the 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 (Figure 5).

3.4 Survival analysis

A strong negative relationship was identified between lesion count and survival duration, where an increase in one lesion per average cross section increased the hazard rate by 1.06 (Hazard ratio: 1.06; 95%CI: 1.03-1.09). The average lesion count per cross section was 27 lesions. Our model predicted that increasing lesion count by 27 on the average cross section would increase the hazard rate 5.3 times. This strong relationship persisted after accounting for *Bsal* infection intensity at necropsy, despite increasing *Bsal* infection intensity also strongly increased the hazard rate of individuals (Hazard ratio for an increase in 1000 zoospore equivalents: 1.01, 95% CI: 1.005 – 1.03). Overall, this analysis

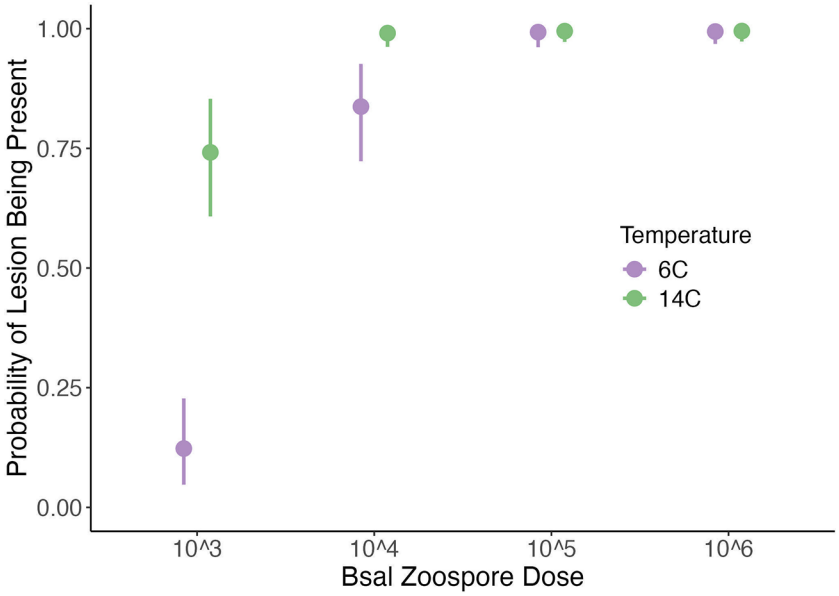

FIGURE 4
Plot of the probability of histologic lesions being present (y-axis) across each *Batrachochytrium salamandrivorans* (*Bsal*) zoospore exposure dose (x-axis) and temperature (6°C = purple; 14°C = green) treatment combination in *Bsal*-infected *N. viridescens*. Error bars are showing the 95% credible intervals around the predicted response.

shows that histological lesions describe variability in host survival beyond infection intensity. Including temperature as an interactive effect with lesion count and *Bsal* load did not significantly affect the model fit.

3.5 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 $5x10^3$, one $5x10^4$, two $5x10^5$, and one $5x10^6$ individual(s). For the 14 °C animals, dermal gland invasion was identified in 1–4 dermal glands in two $5x10^4$, one $5x10^5$, and two $5x10^6$ individual(s). In all regions of dermal gland invasion by *Bsal* organisms, there was accompanying cellular damage affecting the overlying dermis and epidermis.

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*. This study is unique in that it is the first study to evaluate *Bsal* disease progression in infected hosts using histologic evaluation of the number, distribution, and severity of lesions at different temperatures. Environmental temperature as well as *Bsal* zoospore exposure dose were both shown to have a significant effect on histologic lesion development. *N. viridescens* exposed at 22°C did not become infected at any *Bsal* zoospore exposure dose. *N. viridescens* exposed at 14°C had increased lesion counts compared to those exposed at 6°C across all zoospore exposure doses. These findings support those from a previous study (Carter et al., 2021), 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. 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 (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 resistance at 14°C. A similar trend was also identified in a separate study involving *S. salamandra* (Stegen et al., 2017). Our results build on this result in two ways. First, our results show that reductions in lesion counts for a given *Bsal* load have significant positive effects on host survival, indicating that host control of lesions is a mechanism of tolerance. Second, we found that the joint effect of temperature and lesion count on newt survival was near zero. In general, this result suggests that while temperature directly affects host resistance (i.e., reductions in load), it does not affect the

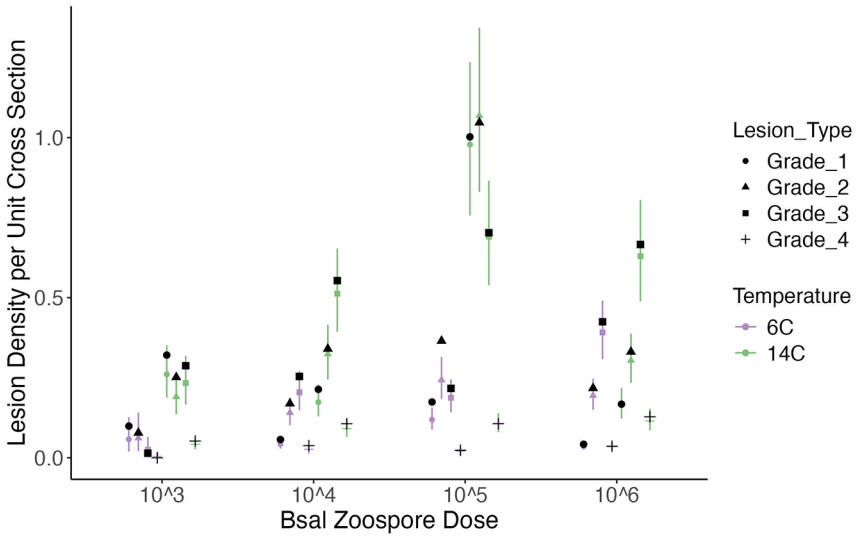

FIGURE 5
Plot of the histologic lesion density per unit of cross section (y-axis) for each *Batrachochytrium salamandrivorans* (*Bsal*) zoospore exposure dose (x-axis) and temperature (6°C = purple; 14°C = green) treatment combination in *Bsal*-infected *N. viridescens*. Each point within a zoospore exposure 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 exposure dose and temperature. Error bars are showing the 95% credible intervals around the predicted response.

strength of this potential form of tolerance. Note that changes in *Bsal* load at 6°C compared to 14°C are also related to the thermal niche of the *Bsal* isolate used in this study in addition to resistance mechanisms of the host. However, the tolerance mechanism we identified (i.e., reducing lesions increased host survival) is independent of temperature and conditional on *Bsal* load, suggesting that this mechanism is largely a property of the host and not the pathogen.

Carter et al (Carter et al., 2021). identified another potential 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. Carter et al (Carter et al., 2021). determined these proteins 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 6 degrees C compared to 14°C may also support this hypothesis. By incorporating histologic lesion counts in this study, we have gained an understanding of disease progression that Carter et al (Carter et al., 2021). was not able to assess without the use of histology. Host microbiome shifts and immune function differences between the two temperatures may have also influenced the disease progression. Carter et al (Carter et al., 2021). also found differences in richness and community structure *on N. viridescens* housed at 6, 14 and 22°C. Immune gene expression at varying temperatures should be explored further.

In regard to changes in the pathogenicity of the fungus, the strain of *Bsal* used in this experiment has an optimal *in vitro* growth temperature 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 (Carter et al., 2021). also confirmed no infection when *N. viridescens* were exposed to *Bsal* at 22°C. It is important to note that not all strains of *Bsal* have the same thermal niche (Kelly et al., 2024), therefore, the results presented here should not be generalized across infection with all strains of *Bsal*. Future studies infecting hosts with additional strains of the pathogen at varying temperatures based on the thermal niche of the pathogen and the host would be needed to explore these potential differences.

The probability of lesion presence being equal between 6°C and 14°C at the two highest *Bsal* zoospore exposure 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 exposure 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 exposure dose combinations, grade 5 lesions were the rarest, followed by grade 4 lesions. These lesion grades occurred most at the higher zoospore exposure 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 exposure 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 exposure dose had the greatest expected lesion density per cross section. This is an interesting finding as the highest zoospore exposure 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 $5x10^5$ exposure dose, the lesions were more commonly less severe than those present in the $5x10^6$ animals. For example, 20 of the 63 grade five lesions were found in $5x10^6$ animals. Therefore, the $5x10^6$ 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, after accounting for *Bsal* intensity (Wilber et al., 2021). This highlights the importance of incorporating histology into *Bsal* chytridiomycosis studies to fully understand the impact of infection and the mechanisms underlying resistance and tolerance (Thomas et al., 2018).

Invasion of dermal glands with *Bsal* zoosporangia was identified in at least one *N. viridescens* from the majority of zoospore exposure 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 (Sheley et al., 2023), as this does not occur in cases of *Bd* chytridiomycosis (Longcore et al, 1999; Berger et al., 2005). Carter et al (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 inflammatory response and were considered incidental. Lack of internal lesions may provide 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; Sheley et al., 2022). 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. Alternatively, skin lesions may create a portal of entry into the body for secondary bacterial infections which lead to bacteremia and contribute to mortality. No lesions associated with bacteremia were present in internal organs; however, in acute cases of bacteremia lesions may not have time to manifest histologically. Although no significant invasion of the epidermis by bacteria was noted in these animals on routine histologic staining with H&E, future studies may incorporate special staining for bacteria such as a Gram stain to highlight bacteria within the lesions histologically. Also, sterile culturing of internal organs could be useful in determining if acute bacteremia may play a role in morbidity and mortality in these infected animals.

Overall, findings show that histologic lesion count and grade are influenced by both environmental temperature as well as zoospore exposure dose in *Bsal*-infected *N. viridescens*. Additionally, these results provide support for the pathogenesis of chytridiomycosis primarily involving damage to the epidermal cells (Sheley et al., 2023). Also, further evidence that dermal gland invasion could potentially play a role in *Bsal* chytridiomycosis disease pathogenesis was provided (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 exposure dose on histologic lesion count in additional amphibian species, as each species responds differently to infection (Martel et al., 2014; Gray et al., 2023). 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, plasma protein electrophoresis and three-dimensional soft tissue imaging would be useful to better understand what disturbances are occurring in the host and leading to morbidity and mortality associated with skin lesions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by University of Tennessee Institutional Animal Care and Use Committee (protocol #2623). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. MW: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. EC: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. MG: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Resources. DM: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Resources.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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