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INNATE IMMUNE FUNCTION IN LAKE ERIE WATERSNAKES (*NERODIA SIPEDON INSULARUM*) WITH OPHIDIOMYCOSIS

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ABSTRACT: Ophidiomycosis, caused by the fungus *Ophidiomyces ophidiicola*, poses a threat to the health of wild and managed snakes worldwide. Variation in snake innate immunity, the primary defense against infection in reptiles, may explain the observed variation in ophidiomycosis clinical disease severity among snakes. In this study, two components of the innate immune response were examined in snake plasma. We investigated whether complement activity, as measured by sheep red blood cell hemolysis, and chitotriosidase activity were associated with ophidiomycosis disease severity and time in captivity in Lake Erie watersnakes (*Nerodia sipedon insularum*). There was no difference in complement-mediated hemolysis or chitotriosidase activities between snakes with varying levels of ophidiomycosis clinical severity sampled in the field. However, among snakes with skin lesions kept in captivity, chitotriosidase activity was significantly higher in snakes with mild disease, compared with snakes with severe disease, and hemolysis activity increased with time in captivity. Overall, Lake Erie watersnakes had higher complement activity, but lower chitotriosidase activity, compared with other reptile species. To our knowledge, this study is the first to describe chitotriosidase activity in a snake species. These results provide mixed evidence of associations between innate immune function and ophidiomycosis severity, and more work is needed to investigate differences among snake species.

Key words: *Ophidiomyces ophidiicola*, ophidiomycosis, Lake Erie watersnakes, innate immune function, complement, chitotriosidase, *Nerodia sipedon insularum*.

INTRODUCTION

Snake health is critically understudied at both the individual and population levels, with only 3% of species evaluated by the International Union for Conservation of Nature (Todd et al. 2010). Threats to snake health include unsustainable removal, habitat destruction, environmental contamination, climate change, invasive species, and infectious disease (Todd et al. 2010). One important infectious disease in snakes is ophidiomycosis, which experimental infection studies indicate is caused by the fungus *Ophidiomyces ophidiicola* (Allender et al. 2015a; Lorch et al. 2015; McKenzie et al. 2020b). Ophidiomycosis has been documented in wild snakes in North America and Europe (Lorch et al. 2016; Franklinos et al. 2017; Meier et al. 2018; McKenzie et al. 2020a) and in managed snakes in Australia (Paré and Sigler 2016), Europe (Vissiennon et al. 1999; Picquet et al. 2018), and the US (Lorch et al. 2016). All species of snakes appear to be susceptible (Burbrink et al. 2017), and the disease has been linked to population declines (Clark et al. 2011) and found in species of conservation concern (Allender et al. 2011; Chandler et al. 2019). Ophidiomycosis typically presents as skin lesions, but there have been reports of systemic disease and mortality in infected snakes (Allender et al. 2011; Dolinski et al. 2014; Ohkura et al. 2016; Robertson et al. 2016; Baker et al. 2019b). Skin lesions can present as one or a few displaced or thickened scales, necrotic scales, larger crusts, or ulceration; they may be present anywhere on the body, and when more severe dermatitis and cellulitis are present, there may be local swelling or structural deformation (Baker et al. 2019b). In some snakes with ophidiomycosis, lethargy, poor body condition, difficulty

shedding, or increased frequency of shedding are also observed (Baker et al. 2019b).

The role of host immune function in the development of ophidiomycosis is poorly understood, and it has been suggested that infection occurs in snakes with compromised immune systems (Lorch et al. 2016). Innate immune function was measured in free-ranging pygmy rattlesnakes (*Sistrurus miliarius*) through plasma bactericidal activity and was found to vary seasonally but did not differ among snakes with a range of ophidiomycosis disease severities (McCoy et al. 2017). The innate immune response, the primary defense against infection in reptiles, is nonspecific and occurs quickly after exposure to a pathogen, whereas the second branch of the immune system, the adaptive response, is pathogen-specific and takes more time to develop. The innate response consists of a variety of cells and proteins, including nonspecific leukocyte responses, lysozymes, antimicrobial peptides, and complement (Rios and Zimmerman 2015). Complement activity has been studied in a variety of reptiles through bacterial killing assays (Merchant et al. 2003; Baker and Merchant 2018a) and sheep red blood cell lysis assays (Merchant et al. 2005, 2010, 2012, 2013; Merchant and Britton 2006; Baker and Merchant 2018b; Baker et al. 2019a). Furthermore, the mammalian innate immune response to fungal pathogens has been found to involve the enzyme chitotriosidase, which targets the chitin found in the cell walls of protozoan parasites, fungi, and nematodes (van Eijk et al. 2005). Specifically, increased levels of chitotriosidase have been detected in the plasma and urine of human neonates with fungal infections (Labadaridis et al. 2005). The activity of this enzyme has been documented in plasma of American alligators (*Alligator mississippiensis*; Kidder et al. 2018) and the broad-snouted caiman (*Caiman latirostris*; Siroski et al. 2014) but has not been measured in any snake species or in any reptiles with active fungal infection.

Lake Erie watersnakes (LEWS; *Nerodia sipedon insularum*) are a subspecies of the Northern watersnake found only in the island region of western Lake Erie (King et al. 2006). Ophidiomycosis was first identified in this population in 2009 (Lorch et al. 2016), and according to yearly disease and pathogen surveillance since 2017, up to 70% of snakes have apparent ophidiomycosis, which is defined as the presence of skin lesions and detection of *O. ophidiicola* DNA by quantitative (q)PCR (Haynes et al. 2022). During these surveys, a wide variety of clinical presentations have been observed, ranging from a few necrotic scales to large areas of crusting, ulceration invading into the deeper tissues of the face and head, or both. Currently, no mechanisms explain the variation in disease severity, but the host immune response has largely been ignored. Therefore, the objective of this study was to measure innate immune function, specifically complement and chitotriosidase activities, in wild LEWS with a range of ophidiomycosis severities. We hypothesized that snakes with more severe disease would have lower immune function and that immune function would decline over time in snakes kept in captivity.

MATERIALS AND METHODS

Animal capture and sample collection

Free-ranging LEWS were captured in visual encounter surveys on Kelleys Island, Erie County, Ohio, US (41°36′48″N 82°42′11″W) and South Bass Island, Ottawa County, Ohio, US (41°39′27″N 82°49′35″W), during the annual population census in June 2019. Each snake received a visual examination for lesions suggestive of ophidiomycosis and was assigned an ophidiomycosis severity score according to the number, type, size, and location of lesions (Table 1), as previously described (Baker et al. 2019b). Each snake received up to 3 points in each of these four categories, for a maximum score of 12 points. Snakes with scores between 1 and 6 were classified as mildly affected, scores between 7 and 9 were classified as moderately affected, and scores of 10 or greater were classified as severely affected. Next, a full-body swab was collected with a cotton-tipped applicator by swabbing the ventrum, dorsum, and lateral aspects of the snake's body, for a total of eight passes along the length of the body. Snakes with skin lesions present also had swabs collected from up to eight individual lesions. Each swab was placed in a separate 2-mL microcentrifuge tube and stored at –20 C until processing. Finally, approximately

TABLE 1. Ophidiomycosis disease severity scoring system adapted from Baker et al. (2019b). Individuals received between 1 and 3 points in each of the four categories. Lake Erie watersnakes (*Nerodia sipedon insularum*) with fewer than 6 points were considered mildly affected, snakes with 7 to 9 points were moderately affected, and snakes with 10 or more points were considered severely affected.

Category	1 point	2 points	3 points
1. Lesion type	Displaced or necrotic scales	Scab, crust, or granuloma	Ulcer
2. Lesion location	Body or tail	Head (except mouth or nasolabial pits)	Mouth, nasolabial pits, or cloaca
3. Lesion no.	1	2–4	5+
4. Lesion length (mm)	<10	10–50	>50

0.5 mL of blood was drawn from the ventral tail vein of each snake with a 23-gauge needle and a 1-mL syringe, then placed in a plasma separator Microtainer® with sodium heparin (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). Blood tubes were stored on ice packs for up to 2 h after collection, then centrifuged for plasma separation (3,500 × G for 10 min). Plasma was stored in cryogenic vials at –80 C until analysis. Thirty snakes with ophidiomycosis were transported to the University of Illinois Urbana–Champaign for a clinical trial, and blood was drawn at two additional time points: after 20 d in captivity and after 70 d in captivity. All snake handling was conducted following the guidelines of the Ohio State University Institutional Animal Care and Use Committee (protocol 2013A00000106-R2) and the University of Illinois Institutional Animal Care and Use Committee (protocol 18163).

Extraction of DNA and qPCR

Extraction of DNA and qPCR amplification were performed to detect *O. ophidiicola* DNA in swab samples as previously described (Allender et al. 2015b). Extraction of DNA followed the manufacturer's recommendations (QIAamp DNA Mini Kit, Qiagen Inc., Valencia, California, USA) with the addition of a 1-h incubation at 37 C with 12.5 units of lyticase (Sigma-Aldrich, St. Louis, Missouri, USA) before the lysis step to break down the fungal cell wall. After DNA extraction, each sample was assessed for DNA quantity (measured in ng/μL) and quality (at the ratio of absorbance at 260–280 nm) by spectrophotometry (NanoDrop 1000, ThermoFisher Scientific, Wilmington, Delaware, USA). We performed the qPCR in triplicate on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Samples were considered positive if replicates had a mean cycle threshold value lower than the lowest detected standard dilution on the same plate.

Innate immune function assays

The functionality of the snake plasma complement system was investigated with a sheep red blood cell (SRBC) lysis assay modified from a previously published method (Mayer 1961). Snake plasma (10 μL) was diluted with 90 μL of saline, followed by the addition of 100 μL of 2% (v/v) unsensitized SRBCs. The reaction was allowed to proceed for 30 min at ambient temperature, then the samples were centrifuged at 2,500 × G, and 150 μL of each of the resulting supernatants was removed to a 96-well plate. The optical density of each sample was determined at 540 nm (OD_{540}) with a Benchmark Plus microtiter plate reader (Bio-Rad, Hercules, California, USA). The OD_{540} for each sample was compared with that of a solution of 1% SRBCs that had been completely hemolyzed by repeated passage through a fine-needled (tuberculin) syringe (Becton, Dickinson and Company). For each sample, the raw hemolytic activity was transformed and expressed as a percentage of the maximum activity.

Chitotriosidase activities were determined as previously described (Argüello et al. 2008). Briefly, 20 μL of plasma were added to 100 μL of assay buffer (150 mM citrate-phosphate buffer, pH 5.2), and the reaction was initiated by the addition of 25 μM 4-methylumbelliferyl-chitotrioside dissolved in 100 μL of assay buffer. After 15 min, 1.0 mL of stop buffer (3 M glycine-NaOH buffer, pH 10.6) was added. The fluorescent product was measured in a FluoroMax-4 fluorometer (Horiba Instruments Inc., Edison, New Jersey, USA) at an excitation λ of 365 nm (slit width 2 nm) and an emission λ of 450 nm (slit width 2 nm). The fluorescent intensities for each sample were corrected for background by the subtraction of fluorescence of snake plasma diluted in assay buffer devoid of substrate. The resulting values were compared with fluorescence values of a standard curve of pure product (4-methylumbelliferone), and the results were expressed as nanomoles of product formed. A positive control of alligator plasma was used to

confirm the functionality of the assay on the basis of its previously documented high chitotriosidase activity (Kidder et al. 2018).

Statistical analysis

Generalized linear regression models were used to predict complement activity (% maximum activity) and chitotriosidase activity (nmol product formed) at capture on the basis of disease severity (no lesions, mild, moderate, severe), site of collection, and sex. Univariable models were created using the *glm* function in R (Venables and Ripley 2002). Post hoc tests were performed using the contrast function in the R package *lsmeans* (Lenth 2016) with a Tukey adjustment for multiple statistical comparisons.

Mixed effects linear regression models were then used to predict complement and chitotriosidase activities in snakes with ophidiomycosis on the basis of disease severity (mild, moderate, severe), time since capture (0, 20, or 70 d), sex, and site of capture. These models were created using the function *gls* in the R package *nlme* (Venables and Ripley 2002). Model sets were created to examine the individual, additive, and interactive effects of predictors for each of the response variables; a repeated measures term was included in all models to account for the nonindependence between samples collected from the same snakes at multiple time points. Models were evaluated by an information theoretic approach, with models ranked according to their second-order Akaike information criterion (AICc; Anderson et al. 2001). For the top-performing model in each set, pairwise comparisons were conducted using the *lsmeans* package (Lenth 2016) with a Tukey adjustment. All analyses were conducted in RStudio version 1.2.1335 (R Development Core Team 2018), and statistical significance was assessed at α=0.05.

RESULTS

Blood was collected from 41 snakes at capture, from 26 snakes after 20 d in captivity, and from 24 snakes after 70 d in captivity. At capture, 11 snakes had no skin lesions, 10 were mildly affected, 10 were moderately affected, and 10 were severely affected. Absence of skin lesions did not always correspond to absence of *O. ophidiicola* DNA on the skin: six of 10 animals without lesions tested qPCR positive. All of the snakes with skin lesions were qPCR positive for *O. ophidiicola*.

Hemolysis activity in blood collected at capture was determined for 41 snakes across four levels of ophidiomycosis disease severity (Fig. 1). From generalized linear regression modeling, SRBC hemolysis was not significantly different between snakes without lesions or with mild, moderate, or severe disease ($P>0.05$). Chitotriosidase activity was determined for the same 41 snakes at capture (Fig. 2), and the generalized linear regression modeling found no significant differences in chitotriosidase activity between snakes without lesions or with mild, moderate, or severe disease ($P>0.05$). There were no associations between either measure of immune function and either sex or site ($P>0.05$).

Hemolysis of SRBCs was determined at capture and after 20 and 70 d in captivity for snakes with mild, moderate, and severe ophidiomycosis (Fig. 3). The top mixed effects linear regression model predicting complement activity was moderately well supposed (Akaike weight, 0.4237) and included the fixed effect of time, as well as the repeated measures factor for snake ID (Table 2a). The model including the additive effects of time and severity, was within two ΔAICc units but the model including the effect of severity alone performed worse than the null model, so severity was likely an uninformative parameter in this model set (Arnold 2010). Hemolytic activity tended to increase over time and was significantly lower at capture (mean, 73.3%), compared with after 70 d in captivity (mean, 86.2%; P=0.0417).

Chitotriosidase activity was determined at capture and after 20 and 70 d in captivity for snakes with mild, moderate, and severe ophidiomycosis (Fig. 4). The top mixed effects linear regression model predicting chitotriosidase activity was moderately well supposed (Akaike weight, 0.5561) and included the additive effects of disease severity, site of capture, and sex, as well as the repeated measures term for snake ID (Table 2b). From this model, chitotriosidase activity was significantly higher in mildly affected snakes (mean, 12.27 nmol) compared with severely affected snakes (mean, 4.98 nmol; P=0.0132). There were no significant pairwise comparisons by capture site or sex based on this model.

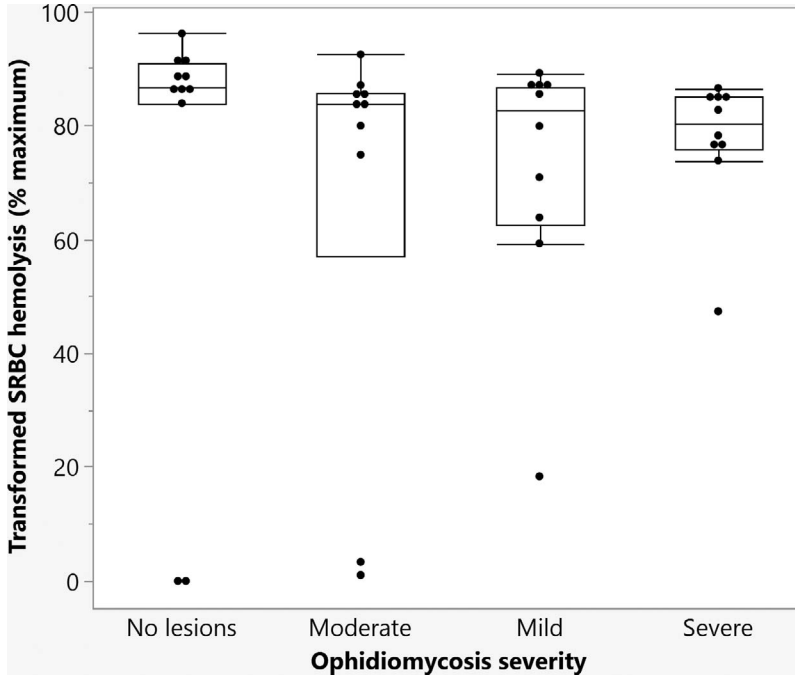

FIGURE 1. Box and whisker plots of transformed sheep red blood cell hemolysis (percentage of maximum) between Lake Erie watersnakes (*Nerodia sipedon insularum*) without skin lesions and with mild, moderate, and severe ophidiomycosis in 2019.

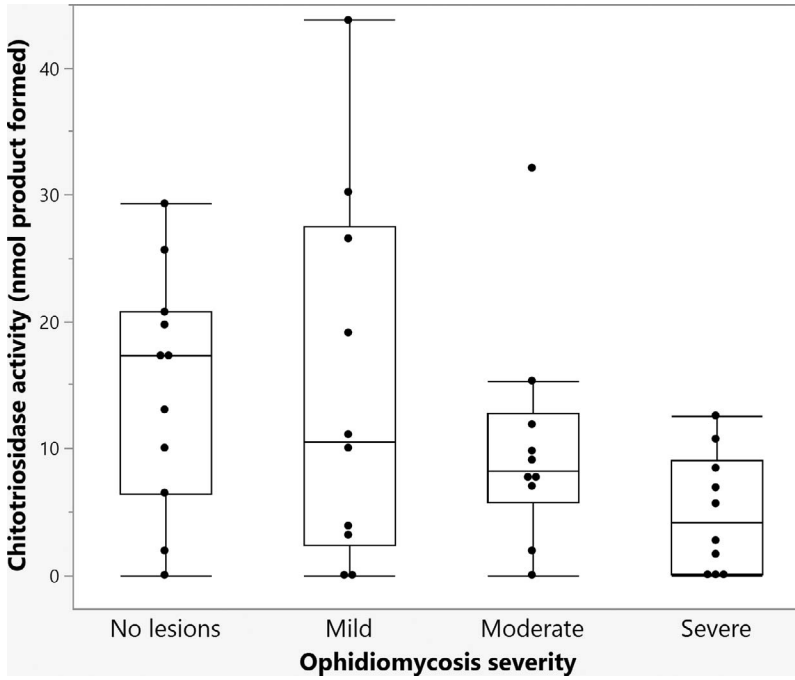

FIGURE 2. Box and whisker plots of chitotriosidase activity (nanomoles of cleavage product) between Lake Erie watersnakes (*Nerodia sipedon insularum*) without skin lesions and with mild, moderate, and severe ophidiomycosis in 2019.

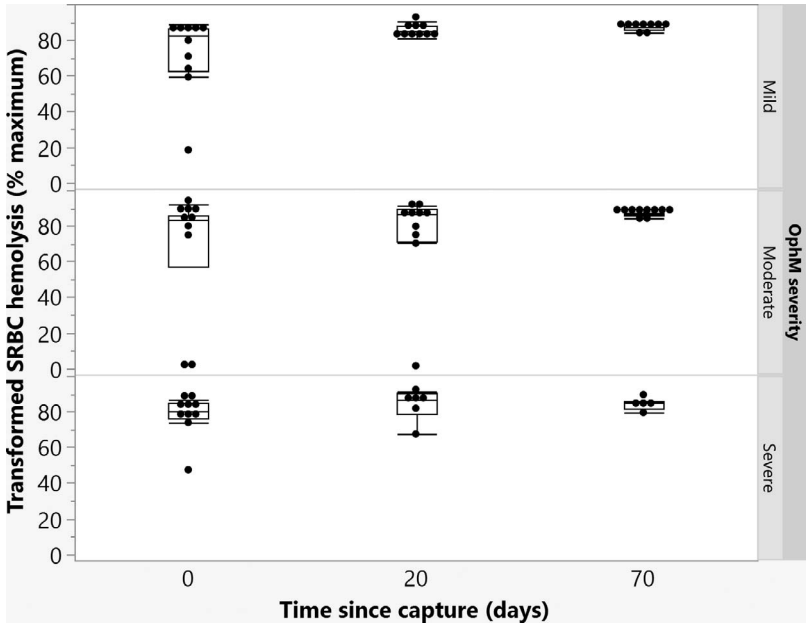

FIGURE 3. Box and whisker plots of transformed sheep red blood cell hemolysis (percentage of maximum) between Lake Erie watersnakes (*Nerodia sipedon insularum*) with mild, moderate, and severe ophidiomycosis at capture after 20 d in captivity and after 70 d in captivity. OphM=ophidiomycosis.

TABLE 2. Second-order Akaike information criterion (AICc) table of generalized linear models predicting (a) complement activity and (b) chitotriosidase activity in Lake Erie watersnakes (*Nerodia sipedon insularum*) in 2019.[a]

Model	K	AICc	ΔAICc	ω_i
(a) Complement activity (% maximum hemolysis)				
Time	5	677.5	0.00	0.4237
Time+severity	7	678.6	1.10	0.2445
Null	3	679.8	2.30	0.1342
Sex	4	681.2	3.70	0.0666
Severity	5	681.4	3.90	0.0603
Site	4	681.5	4.00	0.0573
Time×severity	11	684.4	6.90	0.0135
(b) Chitotriosidase activity (nmol cleavage product formed)				
Site+severity+sex	7	552.3	0.00	0.5561
Severity+sex	6	554.1	1.77	0.2299
Site+severity	6	554.2	1.91	0.2140
Time+site+severity+sex	9	554.3	2.01	0.2032
Site	4	556.8	4.46	0.0599
Site+sex	5	558.2	5.90	0.0292
Severity	5	559.7	7.39	0.0138
Sex	4	560.0	7.72	0.0117
Null	3	560.8	8.49	0.0080
Time+severity	7	561.4	9.09	0.0059
Time	5	563.0	10.69	0.0027
Time×severity	11	564.4	12.09	0.0013

[a] K = no. of parameters; ΔAICc = difference in AICc between ranked models; ω_i = Akaike weight.

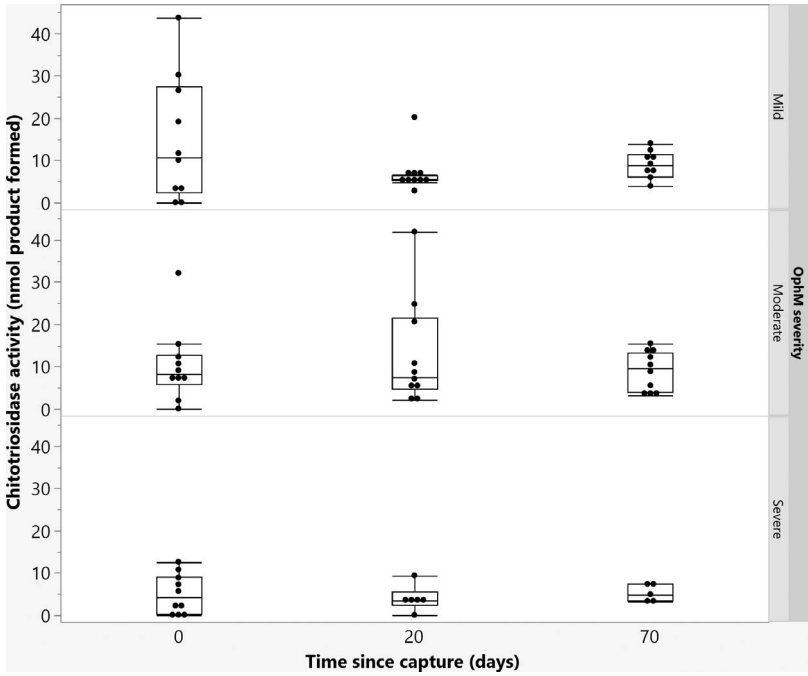

FIGURE 4. Box and whisker plots of chitotriosidase activity (nanomoles of cleavage product) between Lake Erie watersnakes (*Nerodia sipedon insularum*) with mild, moderate, and severe ophidiomycosis at capture after 20 d in captivity and after 70 d in captivity. OphM=ophidiomycosis.

DISCUSSION

This study provides mixed evidence regarding the association between innate immune function and ophidiomycosis severity in LEWS. At capture, neither complement nor chitotriosidase activities differed on the basis of clinical disease severity, sex, or capture site. However, in snakes with lesions kept in captivity, chitotriosidase activity was higher in snakes with less severe disease, and complement activity increased over time. The findings at capture agree with those of previous work examining innate immune function in free-ranging pygmy rattlesnakes with ophidiomycosis, in which the bacterial killing capacity of plasma did not differ among snakes with a range of disease severities (McCoy et al. 2017). Together, these results indicate that snakes with ophidiomycosis do not have reduced innate immune function, as measured by complement and chitotriosidase activities, compared with conspecifics without disease.

Seasonal changes in both disease severity and innate immune function have been observed in pygmy rattlesnakes with ophidiomycosis, with bacterial killing activity lower in the autumn compared with the spring and summer (McCoy et al. 2017), but we found stable or increasing hemolysis and chitotriosidase activities in captive snakes over the time course of this study, which began in the early summer and ended in late summer. The increase in hemolysis activity observed over time in captive LEWS may be due to differences in metabolism between wild and captive snakes, with snakes in captivity having fewer energetic demands and being able to mount a more robust immune response. Snake immune function is temperature-dependent (Rios and Zimmerman 2015), and the temperature gradient provided in captivity may have been more supportive of immune function than the environmental temperatures when the snakes were captured. Factors such as co-infection, environmental contamination, or reproductive status can also affect snakes'

immune status. Lake Erie watersnakes engage in courtship behavior from early May to early June each year (King 1986), which may have been a significant energetic demand for snakes at the time of capture, and two of the female snakes included in this study were gravid, which would be an ongoing energetic demand. Interestingly, chitotriosidase activity did not vary over time, suggesting that these two aspects of innate immune function may be differently affected by systemic physiology.

A difference in chitotriosidase activity between mildly and severely affected animals was only detected in snakes with ophidiomycosis kept in captivity. This finding may be due to the larger number of data points included in this analysis (n=80), compared with the analysis of snakes at capture (n=41), as a result of repeated sampling. This result suggests that there the difference in chitotriosidase activity among disease severity groups might be more subtle, which could become apparent by repeating this study with a larger sample size. Notably, six snakes with lesions died during the course of this study, including five severely affected snakes and one mildly affected snake. Ophidiomycosis disease severity was also found to vary over time, as has been previously described (Lind et al. 2018), which limits the conclusions that can be drawn from disease severity determined at a single time point. However, the use of severity categories spanning a range of disease severity scores allows for some variation in severity over time, and we found that most snakes remained in the same category for the duration of the study.

One important consideration in examining these data is that snakes were captured by visual encounter surveys and cover boards, so we tended to capture snakes that were basking on rocky shorelines or hiding under the cover boards to increase their body temperature. Snakes with ophidiomycosis have been observed to spend more time basking and to move around less, compared with healthy snakes, which may be strategies to offset the energetic cost of the immune response to infection and disease (McBride et al. 2015; Stengle et al. 2017; Tetzlaff et al. 2017). Therefore, the survey techniques used in this study may have biased sample collection toward snakes with ophidiomycosis or other metabolic demands that could affect immune function. However, behavioral thermoregulation is a crucial part of the reptile immune response (Rios and Zimmerman 2015), so snakes found during such activities may have had increased immune function compared with snakes captured during other activities.

The overall complement activity of these LEWS was high compared with published levels for other reptile species. When the plasma samples were diluted to 25%, nearly full hemolysis activity was observed in all samples. The assay was therefore repeated at a 10% dilution of plasma to achieve the results presented earlier. Studies in other reptiles, including introduced wild common (*Chelydra serpentina*) and alligator (*Macrochelys temminckii*) snapping turtles and wild saltwater (*Crocodylus porosus*) and freshwater (*Crocodylus johnstoni*) crocodiles, have found low hemolytic activity with a 10% plasma dilution (Merchant and Britton 2006; Merchant et al. 2010; Baker et al. 2019a). Captive prairie rattlesnakes (*Crotalus viridis*) had approximately 69% maximum hemolytic activity at a 10% plasma dilution (Baker and Merchant 2018b), and captive Komodo dragons (*Varanus komodoensis*) had close to 100% hemolytic activity at this plasma dilution (Merchant et al. 2012). The average hemolytic activity of all LEWS sampled in this study was 78.65% of maximum, and animals without lesions had a mean of 72.62%.

To our knowledge, this is the first investigation and report of chitotriosidase activity in a snake species. The alligator plasma positive control had a mean of 79.6 nmol of product formed, which is comparable to previous reports in this species (Kidder et al. 2018), whereas the LEWS samples had a mean of 9.6 nmol of product formed (range, 0–43.8 nmol of product formed). Chitotriosidase activity also has been investigated in broad-snouted caiman alligators (Siroski et al. 2014), Majorera goats (*Capra aegagrus hircus*; Argüello et al. 2008), and humans (van Eijk et al. 2005), but methodologic differences make it difficult

to compare enzymatic activity among taxa. However, the lower activity of this enzyme in snakes compared with alligators may indicate a lower ability to defend against fungal pathogens, which may explain the high prevalence of ophidiomycosis in LEWS.

Although this study does not provide clear evidence that ophidiomycosis severity is associated with host innate immune function, it is important to continue to investigate the snake innate immune system and its role in overall snake health. In particular, the snake immune response to fungal pathogens is poorly understood and should be investigated at the molecular level through experimental infection studies. More work is also needed to examine differences in immune function across snake taxonomic groups. Ophidiomycosis can affect all snake species, but differences in susceptibility have been described (Haynes et al. 2020). Characterizing innate immune function in additional species may help to explain these differences and the overall epidemiology of this disease.

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