PATHOLOGICAL CHARACTERIZATION OF NATURAL *OPHIDIOMYCES OPHIODIICOLA* INFECTION IN WILD-CAUGHT LAKE ERIE WATERSNAKES: A STANDARDIZED APPROACH TO DOCUMENTATION OF DISEASE

BY

ANDREA E. POHLY

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in VMS- Veterinary Clinical Medicine
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2020

Urbana, Illinois

Advisers:

Clinical Assistant Professor Denae N. LoBato, Chair
Assistant Professor Matthew C. Allender, Co-Chair
Clinical Professor Karen Terio
Professor Lois Hoyer

ABSTRACT

Ophidiomyces ophiodiicola (formerly *Chrysosporium ophiodiicola*), the causative agent of ophidiomycosis (Snake Fungal Disease; SFD), is a serious emerging fungal pathogen causing morbidity and mortality in both wild and captive snakes. SFD has been documented in terrestrial and aquatic captive snake populations in the United Kingdom, Germany, Australia, and North America. The most common presenting clinical signs of SFD infection include severe dermatitis, facial swelling, and emaciation. It was previously thought that lesions associated with SFD were limited to the skin of the head and neck; however, recent reports show well-documented cases of SFD skin lesions throughout many regions of the body. These case reports predominantly focus on grossly visible cutaneous lesions. Rarely, systemic lesions within the lungs, spleen, liver, eye, and kidneys have been documented. SFD can be fatal; however, the complications of this disease leading to mortality, particularly concerning the pathology and role of comorbidities, are poorly understood. This lack of understanding limits the ability and success of clinicians and rehabilitators to treat snakes infected with SFD and inhibits our ability to prevent transmission in both captive and wild settings. Treatment and preventing transmission are particularly important for those species that are endangered or threatened.

The aim of this study was to gain insight into the cutaneous disease severity, subclinical manifestation, systemic involvement, and comorbidities associated with SFD. I hypothesized the following: 1) the systematic mapping protocol derived from this methodology will identify characteristic lesions of SFD in a cohort of wild-caught Lake Erie water snakes, 2) as the number of survey sections affected by SFD increases, so too will the overall cutaneous severity score, 3) histopathological examination will allow for detection of subclinical lesions that are not associated with gross disease, 4) individuals with visceral granulomas will have more severe

cutaneous disease, 5) there will be a range of infectious and non-infectious conditions associated with ophidiomycosis, and finally 6) individuals with decreased visceral adipose tissue will have a higher overall cutaneous severity score as well as head cutaneous severity score than those with adequate visceral adipose tissue.

This study established a detailed necropsy protocol that identified a positive correlation between cutaneous severity and a wider distribution in cutaneous lesions throughout an individual. The results also highlighted a high prevalence (45.3%) of subclinical, or grossly inapparent, cutaneous lesions as well as a lack of correlation between cutaneous severity and visceral granulomas. Finally, this study documented numerous comoribidities, including verminous pneumonia, heterophilic enteritis and splenitis, cutaneous mites, and coccidiosis. The most-prevalent comorbidity was decreased visceral adipose tissue stores which was not associated with cutaneous disease severity. The results of this study suggest that SFD alone is able to cause mortality; however the pathogenesis by which *O. ophiodiicola* does this remains unclear. Additionally, these results provide a process for those studying SFD to evaluate cutaneous severity of disease with clincal (grossly visible) and subclinical (grossly absent) disease.

ACKNOWLEDGEMENTS

First, I would like to thank my primary advisors, Dr. Matt Allender and Dr. Denae LoBato for their support, guidance, and mentorship. Thank you for being patient and supportive throughout this process and working with me as I developed my research skills. I would also like to thank my committee members Dr. Karen Terio and Dr. Lois Hoyer for their willingness to guide me in this research process and provide me with support and direction along the way. Lastly, I would like to especially thank Dr. Ellen Haynes for all of her hard work and help throughout the entirety of my study. This work would not have gotten done without her and I am lucky to have gained not only a wonderful colleague but an even better friend.

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW

Introduction

Ophidiomyces ophiodiicola, the causative agent of Snake Fungal Disease (SFD), is of increasing importance to wildlife conservationists and researchers. Currently, SFD meets the criteria of an emerging infectious disease in snakes, such that *O. ophiodiicola* has been recently observed (Rajeev et al., 2009), infects a wide range of snake species (Burbrink et al., 2017), has ecotypes with high virulence (Allender et al., 2012), and is both an opportunistic and generalist pathogen (Allender et al., 2015d). Although this fungus has likely been present for decades, new understanding of the disease and its clinical signs, as well as increased surveillance, suggest that there has been a rapid increase in the total number of individual snakes affected within the past 2 decades (U.S. Fish and Wildlife Service, 2011). This organism has been implicated as a factor in previous snake population declines and proves to be difficult to control or eradicate in endemic soils (Allender et al., 2016a; Clark et al., 2011; Franklinos et al., 2017). Recently, researchers have focused their efforts to gain a better understanding of *O. ophiodiicola* pathogenesis, molecular characteristics, and possible treatments in the hope of controlling spread of disease.

Background

The first case of *O. ophiodiicola* was noted in 1985 from a captive ball python in England (Paré et al., 2016; Sigler et al., 2013), and this organism has since been documented in over 30 species throughout North America and Europe (Franklinos et al., 2017; Lorch et al., 2016; Sigler et al., 2013). Affected snakes may have varying clinical signs predominantly affecting the integument, but these lesions can progress to widespread, systemic infection that results in mortality at both the individual and population level (Baker et al., 2019; Lorch et al., 2016).

O. ophiodiicola is a member of the order *Onygenales* within the family of *Onygenaceae*. This family contains several fungal pathogens including *Histoplasma capsulatum* and *Coccidioides immitis*. Both of these fungi are well-studied and documented as causing diseases in domestic mammals, particularly canids and felids (Kerl, 2003; Sigler et al., 2013). Initially, *O. ophiodiicola* was thought to be the same species of *Onygenaceae* associated with *Chrysosporium* anamorph *Nannizziopsis vriesii* (CANV) complex, which causes skin lesions such as cutaneous crusting, thickened or raised scales, ulceration, and even necrosis in a variety of squamate reptiles. After full-genome sequencing and comparison between culture characteristics, it is now known that *O. ophiodiicola,* although closely related to CANV*,* is a distinct genus that currently contains only one species (Sigler et al., 2013).

O. ophiodiicola is a saprophytic fungus that is remarkably stable within various environments (Allender et al., 2015d). Although the genome has been sequenced (Ohkura et al., 2017), few studies have attempted to further describe the specific characteristics that allow *O. ophiodiicola* to thrive as a pathogen. Notably, a study performed by Allender *et al.* (2015d) found that *O. ophiodiicola* is able to grow in environments of a wide range of pH (5-11), is able to use multiple complex carbon and nitrogen sources, and is water-stress tolerant. Together, these factors lend further support to this organism's ability to survive in a broad range of microenvironments. This study also found that *O. ophiodiicola* isolates were positive for urease among other enzymes (e.g. gelatinase, β-glucosidase, lipase, lipase/esterase, and keratinase). Urease has been proposed to be a virulence factor in other fungal pathogens; its ammonia biproduct is toxic to host tissues, aiding systemic dissemination (Casadevall, 2003).

In order to identify *O. ophiodiicola* in fungal culture or histopathologically, it is important to understand this organism's lifecycle. Currently, there is no identified sexual state of

this fungus (Rajeev et al., 2009). *O. ophiodiicola* exists in 3 distinct morphologies: hyphae, arthroconidia, and aleurioconidia (Rajeev et al., 2009; Sigler et al., 2013). Fungal hyphae are the main mode for vegetative growth for *O. ophiodiicola*, whereas arthroconidia and aleurioconidia represent asexual reproductive states of the organism (Rajeev et al., 2009). Arthroconidia are produced by segmentation of preexisting fungal hyphae (schizolytic dehiscence) (Sigler et al., 2013), and aleurioconidia are produced by lysis of the supporting fungal hyphae (rhexolytic dehiscence) (Rajeev et al., 2009). It is through these two asexual morphologies that the organism is presumed to initially infect the snake host and initiate cutaneous disease (Rajeev et al., 2009). Initial infection may occur via contact with soil containing *O. ophiodiicola* or from direct or indirect contact with individual snakes that have cutaneous ophidiomycosis, whether they are subclinical carriers or affected individuals.

All three morphologies can be identified in both fungal culture and within tissues, both cytologically and histologically. In culture, *O. ophiodiicola* colonies are yellow-white and moderately fast growing. Hyphae are narrow, branched, and septate, and occasionally form racquet mycelia. Aluerioconidia can be sessile or borne on short stalks and are hyaline, smooth, and cylindrical. Arthroconidia are formed in chains via fragmentation of hyphae, and can be terminal (on the end of hyphae) or intercalary (within hyphae) (Sigler et al., 2013). Histologically, fungal hyphae are up to 5 µm in diameter, have parallel walls, are septate with variably present terminal bulbous dilations, exhibit frequent acute-angle branching, and have transverse septa (Baker et al., 2019). Histologically, fungal aleurioconidia are 2-8 µm x 2-3 µm and are negatively staining (Ohkura et al., 2016) and fungal arthroconidia are approximately 2 x 4 µm, cylindrical, and tend to form aggregates along the skin surface at the air-tissue interface (Baker et al., 2019; Steeil et al., 2018). It is this arthroconidial form, which can be distinctly

identified on routine histopathology, that allows confirmation that *Ophidiomyces ophiodiicola*, as opposed to other fungi, are present in reptilian skin (Baker et al., 2019). Additional histochemical staining, including Gomori methenamine silver and periodic acid-Schiff, can be useful in highlighting distinct fungal morphology.

Epidemiology

O. ophiodiicola has been definitively isolated in over 30 snake species, including those with and without evidence of clinical disease; both captive and free-ranging snakes throughout North America have been affected (Baker et al., 2019). As of 2015, the known United States distribution of *O. ophiodiicola* in native wild snake populations was documented throughout the midwestern and eastern U.S. with cases confirmed in Minnesota, Wisconsin, Michigan, Illinois, Ohio, Pennsylvania, Virginia, Tennessee, Kentucky, South Carolina, Georgia, Alabama, Florida, New Jersey, New York, Massachusetts, and New Hampshire (Allender et al., 2015d). Since then, the fungus has been detected in native free-ranging snakes from additional states, including Louisiana and California, suggesting that this organism has a much wider geographical range than initially presumed. In 2016, a wild juvenile water snake in Louisiana was found inappropriately basking (basking during seasons of brumation) and had severe cutaneous crusting, ulceration, and necrosis as well as emaciation; PCR testing of cutaneous crusts were positive for *O. ophiodiicola* (Glorioso et al., 2016). In 2019, a free-ranging kingsnake in California was found to have severe cutaneous disease as well as emaciation that was attributed to *O. ophiodiicola* via PCR of a whole-body swab (Haynes et al., in press). Within the United States, SFD has also been documented in captive snake populations in Illinois, Virginia, Georgia, Florida, Wisconsin, New York, California, Maryland, New Mexico, and Arizona (Allender et al., 2015). In addition to this organism's prevalence throughout North America, isolates have been

retrieved from macroscopic skin lesions from captive snakes located in England, Germany, and Australia (Franklinos et al., 2017; Sigler et al., 2013). Retrospectively, real-time PCR detected *O. ophiodiicola* on carcasses or molted skin collected from wild snakes in Great Britain and the Czech Republic (Franklinos et al., 2017). This study concluded that *O. ophiodiicola* is widely distributed throughout England and Wales, and is also present in mainland Europe. In this study, however, only the fungal organism was identified, not cutaneous lesions. Therefore, this retrospective analysis only confirms that *O. ophiodiicola* is present within these regions and does not confirm presence of actual disease.

O. ophiodiicola causes disease across numerous and varied taxa of snakes (Burbrink et al., 2017). SFD has been documented in snakes that inhabit various environments (e.g., aquatic, semiaquatic, terrestrial, fossorial), have differing reproductive mechanisms (viviparous vs. oviparous), activity patterns (diurnal vs. nocturnal), brumation habits (communal vs. solitary), and diets (e.g. primarily consuming vertebrates, other snakes, and predominantly invertebrates) (Burbrink et al., 2017). As in cases of severe emerging fungal pathogens, it is suspected that *O. ophiodiicola*'s broad host range plays a major role in its ability to cause catastrophic effects within host populations (Lorch et al., 2016).

While the exact number of snakes that have succumbed to SFD-related disease throughout the United States is not yet known, its presence and effect on several specific snake populations have been recently documented. In Illinois, SFD has been associated with the population decline of Eastern Massasauga rattlesnakes, which are a threatened species under the Endangered Species Act of 2015 (Allender et al., 2016a). This disease has also been documented in populations of Lake Erie water snakes, Timber rattlesnakes, and prairie kingsnakes (Clark et al., 2011; Department of Defense Legacy Resource Management Program, 2019; U.S. Fish and

Wildlife Service, 2011). Though ophidiomycosis could not be confirmed as the cause of the population decline, the Lake Erie water snake population in the Western Basin Lake Erie Islands dropped by an estimated 18% in a single year following an outbreak of SFD (U.S. Fish and Wildlife Service, 2011). A suspected outbreak in New Hampshire Timber rattlesnakes reduced that population by 50% within 1 year (Clark et al., 2011). These outbreaks illustrated differential disease severity that could have potential detrimental effects on population stability (Allender et al., 2015; Dolinski et al., 2014; Last et al., 2016).

Clinical Signs/Gross Lesions

The most characteristic SFD lesions are associated with the skin. Subtle changes include discoloration, roughened or displaced scales, accelerated ecdysis cycles, and epidermal flaking and crusting (Baker et al., 2019). These lesions can progress to increased epidermal crusting, epidermal erosion/ulceration, and inflammation (predominantly heterophilic) of the epidermis, dermis, and/or subcutis (Allender et al., 2015a, Baker et al., 2019). More-severe cutaneous infections may lead to dermal granuloma formation, increased soft tissue inflammation, and extension of inflammation into surrounding bone (Baker et al., 2019; Lorch et al., 2016). While cutaneous infections can be quite severe and are generally the lesions that are readily and most easily identified in surveyed populations, SFD can cause systemic infection (Baker et al., 2019; Robertson et al., 2016; Steeil et al., 2018). Systemic infection is typically characterized by discrete granuloma formation within the body wall, subcutis, and multiple visceral organs including the eye, liver, lungs, kidneys, intestinal mesentery, gastrointestinal system, and soft tissues of the head, tail, and cloaca (Baker et al., 2019; Lorch et al., 2016).

It should be noted that there are cases of snakes with *O. ophiodiicola* qPCR-positive skin swabs in the absence of gross lesions or other clinical signs of disease. These cases may

represent early or recovering forms of disease or may represent asymptomatic carriers of the fungus (Baker et al., 2019; McKenzie et al., 2019).

Pathogenesis

Because of the fairly recent characterization of *O. ophiodiicola*, there is still much work to be done to discern the underlying mechanism by which this organism causes death. While it was previously thought that *O. ophiodiicola* only infected the skin with predilection for the head and tail (Lorch et al., 2016), it is now known that this organism can cause severe cutaneous disease in any region of the body and is capable of systemic infection (Clark et al., 2011; Tetzlaff et al., 2015). The initial source of infection by *O. ophiodiicola* appears to be through the stratum corneum (Lorch et al., 2015). Although a break in the epidermis facilitates fungal invasion of the site, it is not necessary (Allender et al., 2015a; Lorch et al., 2015). Initial infection within the epidermis causes the host to mount an immune response and early lesions consist of epidermal edema and heterophilic inflammation (Lorch et al., 2015). In mild infections of seemingly healthy snakes, molting interval time is decreased, allowing individuals to rid themselves of the superficial infection (Lorch et al., 2015). Molting is an important host defense mechanism in snakes, where the superficial-most keratin overlying the stratum corneum of the epidermis is shed into the environment, thereby eliminating superficial bacteria and fungi (Lorch et al., 2015). Although increased molting lowers the risk of *O. ophiodiicola* infection, in cases where low numbers of fungi remain in the underlying epidermis after molting, individuals may be at risk for chronic infection (Lorch et al., 2015).

Multiple case studies have documented the presence of SFD-associated granulomas within the subcutis, skeletal muscle, and viscera (Allender et al., 2015d; Lorch et al., 2016; Steeil et al., 2018); however, the pathogenesis of systemic spread remains unknown. Systemic SFD-

associated granulomas that have been documented include those affecting the pancreas, lung, eye, gingival stroma, liver, and spleen (Allender et al., 2011; Dolinski et al., 2014; Ohkura et al., 2016; Steeil et al., 2018; Vissiennon et al., 1999). In most cases of subcuticular, intramuscular, or visceral SFD-associated lesions, there are also cutaneous SFD-associated lesions within the same individual. However, in 2018, there was a report of a captive Eastern diamondback rattlesnake (*Crotalus adamanteus*) with multiple intramuscular and subcutaneous granulomas as well as pulmonary granulomas attributable to SFD without associated cutaneous lesions (Dolinksi et al., 2014; Steeil et al., 2018). This case further highlights knowledge gaps in understanding *O. ophiodiicola* dissemination.

Multiple hypotheses have been proposed for how SFD causes death in infected individuals. One hypothesis suggested that severe infection of the head causes facial necrosis and distortion, altering the individual's vision and olfactory sensation and therefore its ability to hunt prey; it is presumed that in these cases, wild snakes die of starvation rather than directly due to SFD infection (Lorch et al., 2016). This hypothesis is supported by the fact that emaciation is commonly seen in wild snakes with SFD, as well as experimentally affected snakes (Last et al., 2016; Lorch et al., 2016; McCoy et al., 2017). However, this hypothesis is contradicted by other work that shows no change in weight despite progressing disease (Guthrie et al., 2016; Picquet et al., 2018). Another hypothesis suggested that SFD infection leads to "risky" behaviors that expose the generally elusive snakes to environmental or predatorial threats (Lorch et al., 2016; Tetzlaff et al., 2017). One example the authors noted was an increase in heat-seeking behavior, whereby snakes are found basking during times when they are typically brumating in order to increase their body temperature to combat infection. This behavior increases their vulnerability to predation and environmental exposure (Lorch et al., 2016). Other hypotheses suggested that

systemic dissemination of *O. ophiodiicola* causes enough tissue damage to lead to host death (Vissiennon et al., 1999) or that death is due to the complications associated with secondary infections, such as bacterial infections (Lorch et al., 2016). While the exact mechanism or mechanisms of death have yet to be determined, it is possible that the mechanism by which SFD causes individual disease and mortality is multifactorial.

Influence of Host Factors on Disease Susceptibility

As previously mentioned, SFD is documented in a wide variety of species. While there is ongoing work aimed at determining if certain species are more susceptible or more-severely affected than others, it is important to note that risk factors and disease spread at the individual and population level are also influenced by environmental, behavioral, genotypic, and phenotypic factors (Burbrink et al., 2017). Simply put, snake species vary not only in their appearance, but also in their natural environment, genetic diversity, and various behavioral traits that likely contribute to disease susceptibility.

An important environmental factor to consider is both the macro- and microclimates in which an individual lives, and how those can impact susceptibility or resistance to various infections. Multiple hypotheses regarding this concept have been proposed. One such hypothesis is that changes in an individual's macroclimate, such as increased rainfall or alterations in temperature and humidity, and variations in soil microbiomes between wet and dry seasons may lead to changes in microbial communities that confer varying degrees of resistance to SFD (Allender et al., 2018). Conversely, cool and wet weather may increase prevalence and severity of fungal disease (Allender et al., 2015d; Lorch et al., 2016). Moist environments also facilitate *O. ophiodiicola's* ability to grow and persist in the environment lending further support to this hypothesis (Lorch et al., 2016). Additionally, hot and/or dry weather may force snakes to spend

time underground, therefore potentially increasing risk of exposure via burrowing through infected soil or by coming into closer contact with affected individuals in the same burrowing locations (Lorch et al., 2016).

Macro- and microclimate also influence snake behavior, host defense, and immune structure. As ectotherms, reptiles must modify their behavior relative to the season and temperature of their surrounding environment (Zimmerman et al., 2010). For example, reptiles are able to raise their body temperature via moving to warmer locations, or water, as well as basking in direct sunlight. While snakes are able to modify their behavior in such a way to compensate for environmental factors, there are physical changes that occur in colder temperatures that the individual cannot control. During colder temperatures, the frequency of molting decreases, allowing pathogens to persist on the skin for longer periods of time. Therefore, it is hypothesized that cool weather may increase the prevalence and severity of snake fungal disease (Allender et al., 2015d; Lorch et al., 2016).

In addition, during cold seasons, reptiles enter brumation to cope with extreme temperatures. During brumation, reptiles burrow in a suitable habitat where shifts in temperature, daylight, humidity, and hormonal changes trigger the individual's heart rate, respiration rate, and metabolism to slow down in order to conserve energy for the length of the cold season (Roman, 2018). During brumation, involution of the thymus and white pulp of the spleen lead to decreased immune activity, possibly predisposing individuals to diseases that they would typically be able to fight off during their active states (Ridi et al., 1981 as cited in Zimmerman et al., 2010).

As previously stated, additional factors that influence host susceptibility to disease include both genotypic and phenotypic characteristics, including population size, habitat

destruction, concurrent disease, and loss of genetic diversity (Allender et al., 2017; Burbrink et al., 2017, Lorch et al., 2016). Small populations may be more affected by morbidity and mortality events because there is a relatively greater population impact when individual animals are lost (Lorch et al., 2016). Habitat destruction limits the area in which snakes can cohabitate, forcing snakes into closer proximity to one another, thereby increasing the chances of direct pathogen spread (Lorch et al., 2016). Second, habitat destruction leading to the direct loss of individuals contributes to smaller population sizes and therefore loss of genetic diversity due to inbreeding of remaining individuals (Allender et al., 2017; Burbrink et al., 2017, Lorch et al., 2016).

Reptilian Immune System

Although the reptilian immune system is vastly understudied, researchers have identified some of its key players. Similar to the mammalian immune system, both innate and adaptive immune responses have been identified in many reptilian species (Zimmerman et al., 2010. The skin and mucosal surfaces are the first lines of innate defense in reptiles (Origgi, 2007). Reptilian skin is often covered in a thick keratin layer that periodically sheds, providing protection against injury and superficial pathogens. If an organism is able to penetrate or evade these initial barriers, reptiles possess factors including transferrin, antimicrobial factors such as lysozymes and "defensin-like" molecules, and the complement system (Origgi, 2007; Zimmerman et al., 2010). Reptiles also have white blood cells similar to their mammalian counterparts, including phagocytes (macrophages, and dendritic cells) as well as natural cytotoxic cells, monocytes, eosinophils, and basophils. Similar to mammals, these cells serve different roles in combatting different types of pathogens. Phagocytic cells, such as monocytes and macrophages, aid in antigen presentation and release cytokines that signal additional cells to the site of infection or

injury (Zimmerman et al., 2010). Heterophils, similar to mammalian neutrophils, are important in fighting off bacterial infections (Zimmerman et al., 2010). Reptilian basophils release histamine in response to an antigen-trigger.

In regard to adaptive immunity, both cell-mediated and humoral immunity have been documented in various reptilian species. Functional T cells, the key players in cell-mediated immunity that are responsible for the regulation of antibody production, have been identified in snakes, lizards, turtles, and tuatara (Burnham et al., 2005). This finding suggests that the reptilian immune system is capable of memory and has the ability to generate rapid immune responses to previously recognized antigens. B-cells, the key players in adaptive immunity and the subset of lymphocytes that is responsible for antibody (immunoglobulin) production, have also been identified in reptiles. However, whereas mammalian B cells produce 5 classes of immunoglobulins, reptiles are currently known to produce at least 2 classes: IgM and IgY (Natarajan, K et al., 1985). IgM is important in combatting Gram-negative bacteria as well as activating the complement system; however, it has a shorter half-life and is produced in small quantities. IgY has a longer half-life and can be produced in greater quantities; however, its specific function is not well-established (Zimmerman et al., 2010).

It is important to note that the reptilian immune system can also vary depending on numerous factors including habitat, life stage, reproductive status, thermoregulatory behavior, and season (Zimmerman et al., 2010). As in most animals, juveniles have a weaker immune system due to the fact that they are in the process of developing their adaptive immune response. Conversely, older individuals tend to have weaker immune responses due to a decrease in the number and function of immune cells. Reptiles in an active reproductive state, such as pregnant females, are also predisposed to certain infections given that their immune system is considered

to be in flux due to the presence of the fetus/fetuses. Given the complexity and seasonal variation in reptilian immunity, its study proves challenging and therefore the system as a whole remains poorly understood.

Variability in Epidermal Structure Between Snake Species

Because SFD causes mild to severe disease in over 30 species of snakes (Franklinos et al., 2017; Lorch et al., 2016; Sigler et al., 2013), it is important not only to assess adaptations of this fungus, but also to understand the variability of disease presentation among species. Documentation of varying disease severities between species suggests that species-specific characteristics may also play a role in disease susceptibility and presentation (Guthrie et al., 2016).

The study of reptilian skin structure and function proves to be a unique and difficult challenge to researchers. Not only does epidermal, dermal, and subcutaneous structure and function vary between snakes from different habitats, but structural characteristics can also vary within an individual depending on age, region of body, and season (Lillywhite et al., 2009; Shine et al., 2019). In a study comparing habitat type to skin thickness, the authors found that many, but not all, sea snakes had 50% thicker skin than their terrestrial and semi-aquatic species (Shine et al., 2019). However, on histologic examination, the authors determined that the epidermis was approximately the same thickness across the differing habitats, and that it was the deep dermis that was of increased thickness in sea snakes (Shine et al., 2019). These variations in dermal thickness and structure may play a role in the differences in susceptibility and severity of SFD infection between species.

Aquatic snakes have significantly less lipogenesis in the alpha keratinizing layer of the epidermis in compared to those of terrestrial snakes (Lillywhite et al., 2009), thereby increasing

water permeability. The epidermis is made up of four distinct layers: the outermost beta layer, the underlying mesos layer, the alpha keratinizing layer, and finally a single germinitive layer (Lillywhite et al., 2009). It is within this alpha-keratinizing layer that production of lipid occurs, which is thought to play a significant role in preventing transepidermal evaporative water loss by creating a hydrophobic barrier (Lillywhite et al., 2009). In aquatic snakes, it is possible that this decrease in lipogenesis within the epidermis relative to terrestrial species, and therefore a decreased hydrophobic barrier, may serve as a mechanism for increased susceptibility to cutaneous pathogens (Lillywhite et al., 2009).

Diagnostic Testing

Currently, the best diagnostic test for detection of *O. ophiodiicola* is real-time quantitative polymerase chain reaction (qPCR) (Allender et al., 2015a; Bohuski et al., 2015) as it has a high degree of sensitivity and analytical specificity. This test specifically targets either the multi-copy internal transcribed spacer region (ITS) or intergenic spacer region (IGS) of *O. ophiodiicola* (Allender et al., 2015a; Bohuski et al., 2015). There are multiple advantages of this test including detection of as few as 10 copies of the fungal gene segment per reaction, allowing identification of the organism early in disease (Allender et al., 2015a). Using this qPCR assay on skin swabs allows for minimally invasive, rapid detection of fungal DNA presence (Allender et al., 2015b). Other methods of detection include traditional fungal culture and identification on histopathology (Bohuski et al., 2015); however, both of these methods have significant disadvantages. Traditional fungal culture may be difficult as there are numerous commensal fungi found naturally on reptile skin that may not be clinically significant (Bohuski et al., 2015; Allender et al., 2018). Other disadvantages include difficulty isolating the organism within small samples, unreliable identification based on morphological characteristics, and the slow-growing

nature of the organism leading to delayed identification (Bohuski et al., 2015). While histological identification of the organism within affected tissue may be helpful, various fungal organisms such as *Fusarium sp.*, *Geotrichum candidum*, *Sporothrix schenckii, Paecilomyces sp.,* and *Pestalotia pezizoies* (Barber et al., 2016) have fungal hyphae similar to that of *O. ophiodiicola*. The presence of arthroconidia distinguishes *O. ophiodiicola* from these other fungi, and therefore must be present to confirm a diagnosis of SFD histologically (Baker et al., 2019).

Disease Control

There are few options for clinically managing SFD in affected snakes. Currently, most efforts are focused on rehabilitating individually affected snakes (Lindemann et al., 2017; Kane et al., 2017; Lorch et al., 2016) both with antifungal therapy and treatment of secondary and/or concurrent disease processes (Lindemann et al., 2017; Kane et al., 2017; Paré et al., 2016). In cases of severe dermatomycosis whereby there is extensive cutaneous or subcutaneous necrosis or proliferative lesions, debulking or debriding of these lesions has been used in attempt to allow systemic antifungal therapy to reach the affected areas (Paré et al., 2016).

Various antifungal therapies, including voriconazole, itraconazole, and terbinafine hydrochloride have been used in attempt to treat affected snakes; however, these have been variably successful thus far (Kane et al., 2016; Lindemann et al., 2017). Minimum inhibitory concentration (MIC) for itraconazole is 0.5-1 µg/mL for two *O. ophiodiicola* isolates and an MIC for voriconazole is 0.25 µg/mL for two *O. ophiodiicola* isolates (Kane et al., 2016). While both of these drugs were shown to be effective at killing *O. ophiodiicola* in culture, in vivo studies highlight their safety concern. In one study where six cottonmouths (*Agkistrodon* piscivorus) were administered 5 mg/kg of voriconazole via subcutaneous injection, three individuals died within 12 hours (Lindemann et al., 2017). The cause of these deaths was not

confirmed; however, seizure-like activity prior to death suggests neurotoxicity as the cause of death. In this same study, two Eastern massasaugas (*Sistrurus catenatus*) and one timber rattlesnake (*Crotalus horridus horridus*) diagnosed with SFD were treated with voriconazole via administration through implantable osmotic pumps; however, the authors noted that therapeutic plasma concentrations of voriconazole were not consistently reached (Lindemann et al., 2017). Lastly, these authors administered a single dose of itraconazole at 10 mg/kg via cloacal administration to seven apparently healthy free-ranging cottonmouths. Results showed that, again, tissue (kidney, liver, skin) and plasma concentrations of the itraconazole did not meet therapeutic concentrations based on *in vitro* data (Lindemann et al., 2017). Given these results, itraconazole and voriconazole are not currently considered safe or effective for use in snakes with SFD.

Terbinafine hydrochloride is also being studied for its potential use in the treatment of SFD. The MIC for terbinafine of 0.015 µg/mL for two *O. ophiodiicola* isolates (Kane et al., 2016). Not only does this drug have the lowest MIC for combatting *O. ophiodiicola* in culture, but it has also been shown to reach therapeutic plasma levels in vitro via both nebulization and slow-release subcutaneous implantation (Kane et al., 2017). It is because of these desired effects that this drug is currently being studied as a non-invasive treatment method that can be used by both researchers and wildlife rehabilitation staff.

Conclusion

The characterization of SFD, its impact on populations, and its pathogenesis have only fairly recently been studied. As an emerging fungal pathogen in snakes, great strides have been made to understand this organism's structure and function (Allender et al., 2015d; Rajeev et al., 2009; Sigler et al., 2013), geographic distribution (Allender et al., 2015d; Franklinos et al., 2017;

Lorch et al., 2016; Sigler et al., 2013), taxonomy (Burbrink et al., 2017), pathogenesis (Lorch et al., 2015), diagnostic testing (Allender et al., 2015a; Bohuski et al., 2015) and gross and histologic appearance of SFD (Baker et al., 2019). However, much remains unknown or poorly understood. To address some of these poorly understood areas, my study will focus on evaluating the presence of subclinical disease, correlating the presence of gross lesions with severity of cutaneous disease, correlating cutaneous and systemic disease, and documenting comorbidities that may impact disease susceptibility and/or severity in wild-caught Lake Erie water snakes. A better understanding of these factors and how they relate to one another will provide insight into subclinical manifestations of SFD, as well as aid in future sampling protocols for diagnostic testing.

CHAPTER 2: A STANDARDIZED APPROACH TO DETECTION AND SEVERITY OF CLINICAL AND SUBCLINICAL CUTANEOUS AND SYSTEMIC LESIONS IN LAKE ERIE WATERSNAKES NATURALLY INFECTED WITH OPHIDIOMYCES OPHIODIICOLA

Abstract

Ophidiomyces ophiodiicola, the causative agent of ophidiomycosis (Snake Fungal Disease, SFD), is an emerging threat to both captive and free-ranging snake populations. Cutaneous lesions typify this disease process and can widely vary in presentation and severity. In this study, a standard protocol was developed using 23 wild-caught Lake Erie watersnakes to assess six hypotheses aimed at identifying pathologic characteristics, including disease severity and co-morbidities, of this often-fatal disease. SFD-associated cutaneous and visceral lesions were documented and characterized via gross examination and through histopathologic evaluation of 12 standardized skin survey sections using a novel histologic grading scheme. There was a positive relationship between the number of survey sections and overall cutaneous severity score. Subclinical lesions (n=117) were more prevalent than expected and were found in 45.3% of all examined survey sections, including sections that were histologically graded as severe. The presence of visceral granulomas was not associated with overall cutaneous severity. The most prevalent comorbidity was absence of visceral adipose tissue; however, this was not associated with severity. Other comorbidities included verminous pneumonia, heterophilic enteritis and splenitis, cutaneous mites, and coccidiosis. This study provides in-depth documentation of both gross and histologic lesions associated with SFD and provides researchers and pathologists a process to evaluate cutaneous severity of disease in individuals with clinical (grossly visible) and subclinical (grossly absent) disease.

Introduction

Ophidiomyces ophiodiicola, the causative agent of ophidiomycosis (Snake Fungal Disease; SFD), is an emerging fungal pathogen causing morbidity and mortality in both wild and captive snakes (Allender et al., 2015d). SFD has been documented in terrestrial and aquatic snake populations in the United Kingdom, Germany, Australia, and North America (Franklinos et al., 2017; Lorch et al., 2016; Sigler et al., 2013). The most common presenting clinical signs of SFD infection include severe dermatitis, facial swelling, and emaciation (Lorch et al., 2016). It was previously thought that lesions associated with SFD were limited to the skin of the head and neck (Allender et al., 2011); however, recent reports have documented SFD skin lesions throughout many regions of the body (Chandler et al., 2019; Guthrie et al., 2016; Tetzlaff et al., 2015). Rarely, lesions within the lungs, spleen, liver, eye, and kidneys have also been documented (Robertson et al.; Dolinski et al., 2014; Baker et al., 2019). SFD can be fatal; however, the mechanisms that lead to mortality, particularly concerning the pathology and role of comorbidities, are poorly understood. Additionally, a current lack of standardized pathologic assessment and histologic characterization of a wide range of SFD-associated lesions suggests that SFD may be underdiagnosed in both wild and captive populations. This information is particularly important for species that are endangered or threatened.

The aim of this study is to gain insight into the cutaneous disease severity, subclinical manifestation, systemic involvement, and comorbidities associated with SFD testing the following hypotheses: 1) the systematic mapping protocol derived from this methodology will identify characteristic lesions of SFD in a cohort of wild-caught Lake Erie water snakes, 2) as the number of survey sections affected by SFD increases, so too will the overall cutaneous severity score, 3) histopathological examination will allow for detection of subclinical lesions that are not

associated with gross disease, 4) individuals with visceral granulomas will have more severe cutaneous disease, 5) there will be a range of infectious and non-infectious conditions associated with ophidiomycosis, and finally 6) individuals with decreased visceral adipose tissue will have a higher overall cutaneous severity score as well as head cutaneous severity score than those with adequate visceral adipose tissue.

Materials and Methods

Study Population. Twenty-three individual, wild-caught Lake Erie water snakes (*Nerodia sipedon insularun*), consisting of 14 females and nine males, were sampled from an ongoing research study investigating novel SFD treatments. Nine of these snakes received terbinafine nebulization, five received saline nebulization, and nine did not receive any treatment. qPCR of full body swabs confirmed presence of *Ophidiomyces* during ante-mortem (n=19) or post-mortem (n=4) exam. All snakes in this study were either found dead or euthanized due to disease and were submitted for necropsy examination (Table 2.1).

External Exam, Diagnostic Necropsy, and Sample Collection. Measurements were taken from the tip of the snout to the tip of the tail, the tip of the snout to the cloaca, and the base of the skull to the cloaca. Each snake was linearized and nine equal sections (survey sections) were identified from the base of the head to the cloaca (Figure 2.1). Next, each cutaneous lesion, defined as any gross abnormality in scale appearance including hyperkeratosis, roughening, missing scales, scar formation, or retained shed as previously defined in Baker et al. (2019) was documented. Each lesion was assigned a unique identifying letter code, section associated with the lesion was recorded, diameter of the lesion was measured, gross description was recorded, and each lesion was photographed.

The animal was incised along the ventral midline. Any subcutaneous nodules present were documented in the same format described for cutaneous lesions. Gross internal abnormalities, sex, and body condition (presence/absence of fat reserves) were recorded. Individuals with grossly visible visceral adipose bodies were classified as having "adequate" visceral adipose, and those without grossly visible adipose were classified as having "minimal" visceral adipose (Figure 2.2). Fresh samples of the lung, liver, kidney, gastrointestinal tract, and adipose tissue (if identified) were harvested and frozen at -80 °C. Full thickness, circumferential sections of the cloaca, tail, and the nine survey sections throughout the body were collected and fixed with 10% neutral-buffered formalin (Figure 2.3). Additionally, the entire head and sections of all viscera were collected and fixed with 10% neutral-buffered formalin.

Histopathologic Evaluation and Case Definition. Formalin fixed tissues were routinely processed and embedded, and 5 μm sections were cut, mounted on glass slides, and stained with routine hematoxylin and eosin (H&E). Using histopathologic findings including presence or absence of fungal hyphae and/or arthroconidia, along with *Ophidiomyces* PCR testing (see *Molecular Testing*), each individual snake was assigned a classification of “confirmed”, “apparent”, “possible”, or “present” in accordance with the previously published case definition (Baker et al., 2019; Table 2.2).

Individual Cutaneous Lesion Classification and Cutaneous Lesion Severity Scoring. Lesions were classified as “clinical” if both macroscopic and microscopic evidence of disease were observed within the survey section. Lesions were classified as “subclinical” if only microscopic evidence of disease was present. Each section was examined histopathologically and assigned a histologic grade (that correlated with increasing histologic lesion severity) based on a previously published grading scheme (McKenzie et al., 2019) (Table 2.3). Briefly, histologic features were

graded as follows: Grade 1 cutaneous severity lesions were characterized by low numbers of heterophils within the epidermis (Figure 2.4A); Grade 2 lesions included mild hyperkeratosis (Figure 2.4B), mild to marked serocellular crusts containing fungal organisms overlying the epidermis, heterophilic epidermitis, heterophilic, granulomatous, and/or lymphoplasmacytic dermatitis, and/or distinct dermal granuloma formation (Figures 2.4C, 2.4D, and 2.4E); and Grade 3 lesions had at least two of the following: severe epidermal ulceration and serocellular crusts containing fungal organisms (Figure 2.4F), extensive coagulative necrosis of the epidermis, dermis, subcutis, and skeletal muscle (Figures 2.4F and 2.4G), effacement of bone, and/or evidence of bony remodeling (Figures 2.4G and 2.4H), and inflammation extending into the bone marrow spaces (Figure 2.4I). The cutaneous severity grades of the 12 locations were summed for each snake to give a single overall severity score. The maximum overall cutaneous severity grade was 36 (i.e. if all 12 sections had a grade 3 severity).

Molecular Testing. DNA was extracted from the tissues (DNeasy kit, Qiagen, Valencia, CA) and skin swab samples using commercial kits (DNA Blood Mini Kit, Qiagen, Valenica, CA). The DNA extracts from skin and internal organs were assayed for *Ophidiomyces* using qPCR (Allender et al., 2015b) and pan–herpesvirus using conventional PCR (VanDevanter et al., 1996).

Statistical Analysis. Statistical analysis was performed to test the six established hypotheses. Prevalence proportion of snakes in each disease category, sex, and species were tabulated for both clinical and subclinical disease. Pearson's correlation was used to determine the relationship between number of sections affected by SFD and the overall cutaneous severity score. Histologic grade was compared between gross lesion types (e.g. crust, thick scale, raised scale, ulcer, and necrosis) using a Kruskal-Wallis test. The total number of segments with subclinical disease was

compared between snakes that received terbinafine treatment and those that either received saline nebulization or no treatment using a Mann-Whitney U. A Mann-Whitney U Test was also used to compare overall, head, tail, and cloacal severity between individuals with and without visceral granulomas. Similarly, a Mann-Whitney U test was used to compare the overall cutaneous severity scores between individuals with varying amount of visceral adipose tissue stores. Comorbidities were described qualitatively and tabulated. All comparisons were calculated using R statistical software (R core team, 2019). Statistical significance was considered for all p values ≤ 0.05.

Results

Hypothesis 1: Systematic mapping protocol derived from this methodology will identify characteristic gross and histologic lesions of SFD in a cohort of wild-caught Lake Erie watersnakes.

Gross evidence of cutaneous ophidiomycosis was identified in each of the 9 survey sections of the body, as well as the head, tail, and cloaca of at least one snake. Evidence of SFD was not found along the oral or cloacal mucous membranes. Several gross lesion types were observed, including brown friable crusts, raised scales, thickened scales, ulceration, granuloma formation, and necrosis (Figure 2.5A-F, Table 2.4). Crusts were identified throughout each of the 12 survey locations (i.e. there was no location that didn't have at least 1 snake with an SFD-associated lesion at that location). Raised scales were present in each of the survey locations, except the head and cloaca, of at least one snake. Thickened scales were identified in the head, tail, and body survey section locations 2, 3, 4, 5, and 8 of at least one snake. Gross evidence of ulceration, defined as complete loss of scales/epidermis that expose underlying dermis or deeper tissues, was identified in a single snake in body survey section 4; a granuloma was identified in

the head of a single individual; and gross evidence of necrosis, defined as a single or multiple scales that was elevated or misaligned, discolored, and sloughed when light pressure was applied (Baker et al., 2019), was identified in the tail of 2 individuals (Table 2.5). Grossly evident necrosis (median=3) had the most severe histologic grade, followed by displaced/thickened scale (median=2), crust (median=2), and none (median=1) ($p<0.0001$).

A total of 276 survey sections were examined (23 snakes, 12 survey sections per individual). There were 258/276 (93.5%) survey sections that had evidence of SFD-associated lesions ranging from histologic grade 1-3 (Table 2.6). All 23 snakes had histologic evidence of ophidiomycosis in the head as well as at least one lesion within the cranial, middle, and caudal one-third of the body. Twenty-one (91%) snakes had evidence of ophidiomycosis in the cloaca, and 20 (87%) snakes had evidence of ophidiomycosis in the tail (Table 2.7). The median histologic grade for lesions of the head was a grade 3. The median histologic grade for lesions of the tail was a grade 2. The median histologic grade for all remaining survey sections (all 9 body sections and cloaca) was a grade 1 (Table 2.8).

The study population consisted of eight snakes that received terbinafine nebulization treatment for different lengths of time, six that received saline nebulization as a control, and nine that did not receive any form of treatment. Snakes that were treated with terbinafine nebulization had a significantly lower overall histologic cutaneous severity score (median= 13) than those that either received saline nebulization or no treatment at all (median=19) ($p=0.022$, $W=24$).

Hypothesis 2: As the number of survey sections affected by SFD increases, so too will the overall histologic cutaneous severity score.

The total number of sections affected by SFD-associated lesions for each snake ranged from 8-12 (median = 12). Cutaneous severity scores in an individual snake ranged from 9 to 31

(median = 16) (Table 2.9). There was a significant positive correlation between the total number of survey sections with histologic evidence of ophidiomycosis and the cutaneous severity (Table 2.10). Furthermore, histologic grade of survey sections 1, 3, and 7 showed the greatest correlation with overall cutaneous severity (r = 0.869, 0.742, and 0.744 respectively).

Hypothesis 3: Histopathological examination will allow for detection of subclinical lesions that are not associated with gross disease

Subclinical disease was identified in 117 out of 258 survey sections with lesions of SFD (45.3%). Histologic grades ranged from grade 1 to grade 3 in subclinical lesions. Clinical disease was identified in 141 out of 258 survey sections with lesions of SFD (54.7%). Histologic grades of clinical lesions ranged from grade 0 to grade 3. The three sections (out of 258 total sections with lesions of SFD) that were classified as a grade 0 were identified as having a grossly identifiable cutaneous lesion, but no evidence of microscopic SFD-associated disease.Therefore, these lesions were interpreted as unassociated with SFD. In 18/276 (6.5%) total survey sections, there was no evidence of microscopic or gross SFD-associated lesions (Table 2.11).

Snakes that were treated with terbinafine nebulization had a median of six survey sections that were affected by subclinical disease. Snakes that did not receive treatment or received saline nebulization had a median number of five survey sections that were affected by subclinical disease. There was no significant difference in the prevalence of subclinical disease between snakes treated with terbinafine nebulization and those that either received saline nebulization or no treatment at all (p=0.922, W=58).

Following gross, histopathologic, and molecular evaluation, 16 (of 23) snakes were diagnosed with confirmed ophidiomycosis, three were classified as apparent ophidiomycosis, and four were classified as possible ophidiomycosis (Table 2.2).

Hypothesis 4: Individuals with visceral granulomas will have more severe cutaneous disease, as well as a higher median head, tail, and cloaca severity score than those without visceral granulomas.

Subcutaneous granulomas were identified in three (13%) watersnakes. Only one had characteristic fungal hyphae of *O. ophiodiicola* within the center of the subcutaneous granulomas.

Visceral granulomas were histologically identified in 8/23 individuals and were associated with the respiratory system (trachea, lung), gastrointestinal tract (esophagus, stomach, liver), urinary system (kidney), and hematopoietic (spleen) system. Of the eight individuals with visceral granulomas, overall cutaneous severity scores ranged from 11-23. Only two of the snakes demonstrated characteristic *O. ophiodiicola* hyphae within the center of the visceral granulomas, neither of which had subcutaneous granulomas containing intralesional characteristic *O. ophiodiicola* hyphae. Snakes with visceral lesions had no more severe cutaneous infections than those without visceral involvement ($p = 0.234$). Additionally, snakes with visceral lesions did not have more severe cloacal, tail, or head infections than those without visceral involvement ($p = 0.516, 0.897, 0.347$ respectively).

Hypothesis 5: There will be a range of infectious and non-infectious conditions in snakes with ophidiomycosis.

Comorbid skin conditions were documented in all 23 snakes (100%). Comorbidities of the skin included concurrent bacterial infection (100%), algal infection (4%), and cutaneous mite presence (22%). Intraluminal nematodes without inflammation were observed in both the respiratory (78.3%) and gastrointestinal tract (56.5%). Comorbid conditions were found less

frequently within the liver, splenopancreas, esophagus, trachea, central nervous system, heart, kidneys, reproductive tract, and subcutis (Tables 2.12 and 2.13).

Hypothesis 6: Individuals with decreased visceral adipose tissue will have a higher overall cutaneous severity score as well as head cutaneous severity score than those with adequate visceral adipose tissue.

Marked reduction in visceral adipose tissue stores was common, as visceral adipose tissue was not grossly visible in 19 of the examined snakes (82.6%). There were no significant differences in the severity of head lesions between snakes with adequate visceral adipose tissue (n=4) and those with minimal visceral adipose tissue (n=19) ($p=0.075$, $W=58$). There was no significant difference in the overall cutaneous severity between snakes with adequate adipose tissue (n=4) and those with minimal visceral adipose tissue (n=19) ($p=0.745$, $W= 42$).

Discussion

Using our standardized sampling system of surveying circumferential skin sections throughout 12 standardized body sections of each individual, a wide range of SFD-associated lesions were documented that are consistent with previous studies (Allender et al., 2011; Baker et al., 2019; Lorch et al., 2016). Gross lesions of necrosis had the highest histologic grade, which is consistent with known pathogenesis (Baker et al., 2019) and signifies the most severe lesion type. All areas of the body are susceptible to SFD, as demonstrated by the presence of SFD-associated lesions in all survey section locations. Out of the 12 survey locations, the head was consistently the most severely affected and had the fewest subclinical lesions. Early reports of SFD were mainly associated with lesions of the head (Allender et al., 2011); thus, it is reasonable to assume that initial observations of this disease were diagnosed based solely on the most severe, grossly visible lesions of the head (Lorch et al., 2016). Because the head appears to be

the most commonly and most severely affected, the head is a good location to inspect and sample to identify the presence or absence of disease. Additionally, it seems like subclinical lesions are unlikely in the head either due to anatomic differences or possibly due to higher exposure to fungal quantity. Future studies should investigate how location of initial infection and fungal quantity affect disease progression.

It is important to note that histologic lesions of varying severity were often present within the same individual. This finding is likely attributable to multiple factors including cutaneous bacterial coinfection, chronicity of lesion, and predisposing lesions such as trauma. In all 23 snakes, at least one cutaneous lesion per individual that was attributed to SFD also had concurrent intralesional bacterial organisms. The secondary infection likely complicates the snake's ability to resolve the ongoing infection. Additionally, individual snakes had multiple cutaneous histologic lesions that differed in their inflammatory cell components. For example, multiple individuals had survey sections that exhibited predominantly a dermal macrophage infiltrate as well as formation of discrete, dermal granulomas without epidermal inflammation, ulceration, or crusting, suggesting a more chronic cutaneous lesion that would be classified as a grade 1 histologic severity. In these same individuals, other survey sections showed a predominantly heterophilic epidermal and dermal cellular infiltrate with prominent epidermal ulceration and crusting, which suggests an acute or ongoing inflammatory process that would be classified as a grade 2 histologic severity. It is possible that this variation in cutaneous severity may represent a spectrum of early and late stages of disease where lesions are in various phases of resolution. Lastly, previous studies suggest that a break in the epidermis facilitates fungal invasion of the site (Allender et al., 2015a; Lorch et al., 2015). In this study, it is possible that more severe cutaneous lesions represent previous sites of trauma or excoriation that facilitated a

more severe infection. Given that these were wild-caught animals, and therefore without known clinical history, previous damage to the epidermis can only be speculated and further investigation using an experimental design would be necessary to further tests this hypothesis.

Results of this study show, unsurprisingly, that as the number of survey sections affected by SFD-associated lesions increases, the overall cutaneous severity increases as well. This relationship indicates that a wide distribution in lesion location is indicative of more severe overall cutaneous disease. The increasing histologic grade of survey sections 1, 3, and 7 is strongly associated with increasing overall number of sections affected by SFD. Therefore, during clinical or necropsy examination, ensuring that these three survey locations are examined for SFD-associated lesions will be beneficial in determining whether or not the individual has severe cutaneous disease. This information directs those performing necropsies on snakes with potential SFD to be able to extrapolate as much information regarding the snake's overall cutaneous disease without needing to histologically examine numerous samples.

One of the limitations of this study was that the sample population used wild-caught individuals with severe cutaneous SFD of unknown duration. Therefore, these conclusions may not hold true when evaluating less severely affected individuals. Additionally, this study population was composed of a single species. Previous studies suggest that there are species-specific factors, such as skin structure, habitat, and genotypic and phenotypic diversity that play a role in disease susceptibility and presentation (Burbrink et al., 2017; Guthrie et al., 2016; Shine et al., 2019). Thus, future studies using this method should investigate different species and/or natural habitats in snakes with a full spectrum of disease, ranging from mild to severe, in order to evaluate the effects of these various factors on this study's conclusions.

Currently, few studies have documented and characterized subclinical disease (Allender et al., 2015a; Lorch et al., 2015); however, many studies solely focus on grossly visible lesions and tend to describe severe infections associated with SFD (Guthrie et al., 2016; Last et al., 2016; Ohkura et al., 2016). Subclinical disease may represent early or resolving *O. ophiodiicola* infection, as many of these lesions had minimal heterophilic inflammation, suggesting acute inflammation, or had well-defined regions of granulomatous inflammation, suggesting late stage or chronic infection. Documentation and characterization of subclinical lesions is crucial in identifying cases of SFD, characterizing the epidemiology, and implementing successful treatment strategies. Additionally, characterization of subclinical lesions will help researchers and rehabilitators to monitor resolution of SFD infection. It should be noted that there was no significant difference in subclinical disease prevalence between treated and non-treated snakes; therefore, it is possible that current treatment methods may not adequately rid snakes of subclinical lesions.

In this study, 45.3% of SFD-associated lesions identified were identified by histopathology alone and did not show evidence of gross lesions. The current case definition emphasizes the use of a combination of *Ophidiomyces* PCR/fungal culture, gross SFD-associated lesions, and histologic evidence of SFD-associated lesions along with identification of the fungal organism and morphology in confirming the diagnosis of ophidiomycosis (Baker et al. 2019). In this study, not only were subclinical SFD-associated lesions identified, but they happened with such frequency that it calls into question the utility of the current case definition. It is reasonable to conclude that histopathology using circumferential sampling is more appropriate than using gross lesions alone to identify confirmed ophidiomycosis. However, this circumferential sampling is impractical in live snakes. Therefore, future studies using smaller survey samples is

warranted to see if this result holds true. There were also low numbers of grossly visible SFD-like lesions that were documented without microscopic evidence of SFD. This finding indicates that gross lesions that are typically attributed to SFD may sometimes be representative of other disease processes, such as previous trauma, bacterial infection, or to fungal infections other than *O. ophiodiicola*, such as *Fusarium sp.*, *Geotrichum candidum*, *Sporothrix schenckii, Paecilomyces sp.,* or *Pestalotia pezizoies* (Barber et al., 2016). It is also possible that the highly focal nature of this disease failed to include the characteristic lesions of SFD in the plane of section evaluated. Therefore, in snakes with suspicious lesions, it is crucial to histologically examine multiple sections, as evaluation of a single plane of section may fail to identify SFD-associated lesions that are actually present.

The vast majority of subclinical lesions, and even those lesions that were grossly visible, did not have microscopic evidence of the fungal organism. All snakes in this study were confirmed to be qPCR-positive for *O. ophiodiicola*; however, this result was based on a full body swab, not swabs from each individual survey section. Therefore, although lesions were classified as "SFD-associated" according to the current case definition (Baker et al., 2019), it would be necessary to run qPCR and histopathology for *O. ophiodiicola* on each sample to confirm the lesions are directly associated with the presence of *O. ophiodiicola*. It also illustrates the importance of considering SFD in animals without histologically visible fungal organisms.

The pathogenesis of systemic infection by *O. ophiodiicola* has not been established and there is a paucity of information associating cutaneous disease severity and systemic disease. In this current study population, snakes with visceral granulomas did not have more severe cutaneous disease. This finding suggests that either severe cutaneous infection is not associated with systemic visceral granuloma formation, or that visceral granulomas are not due to *O.*

ophiodiicola infection. While fungal organisms within visceral granulomas were identified in only two snakes, it is possible that other organisms such as *Mycobacteria sp.* or other bacteria were the underlying cause of these visceral granulomas. It is also possible that intralesional fungal organisms within granulomas were not included in the plane of section that was evaluated, which highlights the limitation in using only histopathology for diagnosis. If these granulomas are, in fact, due to *O. ophiodiicola* infection, there are a number of confounding factors that may have resulted in falsely concluding that there is a lack of correlation between cutaneous severity of infection and visceral granulomas formation. Two confounding factors present in this study include duration of infection and treatment effect. For example, individuals in this study that were treated with terbinafine nebulization tended to have lower overall cutaneous severity scores than those that were not treated or that received saline nebulization as a control. Thus, this confounding variable of treatment may have contributed to an overall decrease in median cutaneous severity in snakes with visceral granulomas when comparing these scores between snakes with and without visceral granulomas. It remains unclear how visceral granulomas relate, if at all, to disease severity. Although this study's results suggest that there is no correlation between cutaneous severity and visceral granuloma formation, anecdotal evidence suggests that systemic dissemination, or visceral granuloma formation, may be a consequence of fungemia secondary to severe cutaneous infection.

Severe cutaneous infection may lead to fungemia and secondary systemic dissemination. In this study, fungal organisms were not identified within the vasculature, nor was there evidence of a vasculitis. However, given the variability in sample surveillance as well as section of cut of histologic sections, this observation does not completely rule out fungemia as a potential

mechanism of spread. Further studies, such as blood culture, may be necessary to lend further support to this proposed pathogenesis.

The most common comorbidity found within our study population was a marked reduction in visceral adipose tissue. This finding is consistent with previous reports of severe emaciation and starvation in cases of snakes with severe fungal disease (Lorch et al., 2016). It is hypothesized that in these cases, severe infection of the head could alter the individual's vision and olfactory sensation thereby inhibiting its ability to hunt prey (Lorch et al., 2016). There was no difference in the histologic severity of the head in Lake Erie water snakes between the snakes with adequate versus decreased visceral adipose tissue, suggesting SFD-associated head lesions do not contribute to a snake's ability to maintain adequate fat reserves. Alternatively, it is possible that decreased visceral adipose tissue stores are associated with an increase in resting metabolic rate as observed in pygmy rattlesnakes (*Sistrurus miliarius*) (Agugliaro et al., 2019). A decrease in adipose stores may be mediated through a stress response in which energetic priority is given to essential life-preserving functions, such as an increase in cortisol, glucagon, and catecholamines (Ndahimana et al., 2018), or to mount an immune response (Agugliaro et al., 2019; Hawley et al., 2012; Otalora-Ardila et al., 2016). Production of phagocytes, synthesis of immunoglobulins, production of components of the complement system, production of numerous intracellular enzymes, and production of proteins are dependent upon adequate cellular energy (Beisel, 1975). Therefore, it is unsurprising that the energetic resources of SFD-affected snakes are being used to combat infection and thus are consuming any visceral fat stores in the process.

While a direct link has not been established between specific comorbidities and severity of SFD, many snakes within this study population had comorbidities of both infectious and non-infectious etiologies. Within the vast majority of the snakes examined, comorbidities were

considered incidental and did not appear to incite a significant inflammatory response to suggest contribution to the animal's demise. Further investigation into the specific comorbidities is necessary to understand disease pathogenesis and their contribution to the animal's overall health status. However, this study shows that it is likely that SFD alone can lead to individual mortality.

The exact mechanism by which SFD causes mortality is unknown. As previously mentioned, many affected snakes have decreased visceral adipose tissue stores, and many reports describe severe emaciation associated with disease (Lorch et al., 2016; McCoy et al., 2017). It is possible that snakes with SFD, whether with severe cutaneous disease or persistent low-grade chronic infection (i.e. those with low overall cutaneous severity grades), are in a constant state of negative energy balance that ultimately leads to decline of the host's condition and death (Agugliaro et al., 2019). However, the true mechanism leading to mortality is not clear from this or any other study and should remain a focus of future research.

This study provided in-depth documentation of both gross and histologic lesions currently associated with SFD and highlighted the importance of combining fungal PCR/culture, gross and histologic examination to accurately assess the SFD infection status of individual snakes. In particular, this study found that subclinical lesions are nearly as common as cutaneous gross lesions. This information is important for researchers and rehabilitators to be aware of, as gross examination alone cannot rule out *O. ophiodiicola* infection or confirm clearance of *O. ophiodiicola* in treated individuals, and may in fact miss significant SFD lesions. In the same regard, negative *O. ophiodiicola* PCR of cutaneous swabs should be interpreted with caution, as subepidermal or visceral presence of *O. ophiodiicola* can be missed.

This study found that, in this species, the head is often the most severely affected portion of the snake, although subclinical lesions were rarely documented in the head. Because there will

likely be gross lesions if the head is affected by SFD (and thus histology might not be needed for detection of disease in these animals), the head is a poor site for surveillance in animals without cutaneous gross lesions. For more in-depth information on the overall cutaneous severity of disease in an individual, this study shows that the best samples to take include those from survey sections 1, 3, and 7 as the severity in these sections corresponds to overall severity of disease.

Future directions include using this survey method in various snake species including those that differ in natural habitat and location. This information would be beneficial in determining if there is a particular section of the animal that is consistently affected by gross cutaneous or subclinical disease. Secondly, use of this survey method would be beneficial in studies that evaluate response to treatment as this method does not solely rely on gross lesions as an indicator of disease. In particular, biopsy examination of survey sections may be helpful in tracking not only treatment response, but could also be used in tracking disease progression as lesions progress or regress over time.

TABLES AND FIGURES

Figure 2.1. Eastern massasauga rattlesnake (*Sistrurus catenatus*). Twelve survey sections were sampled as part of a standardized gross and histopathologic evaluation.

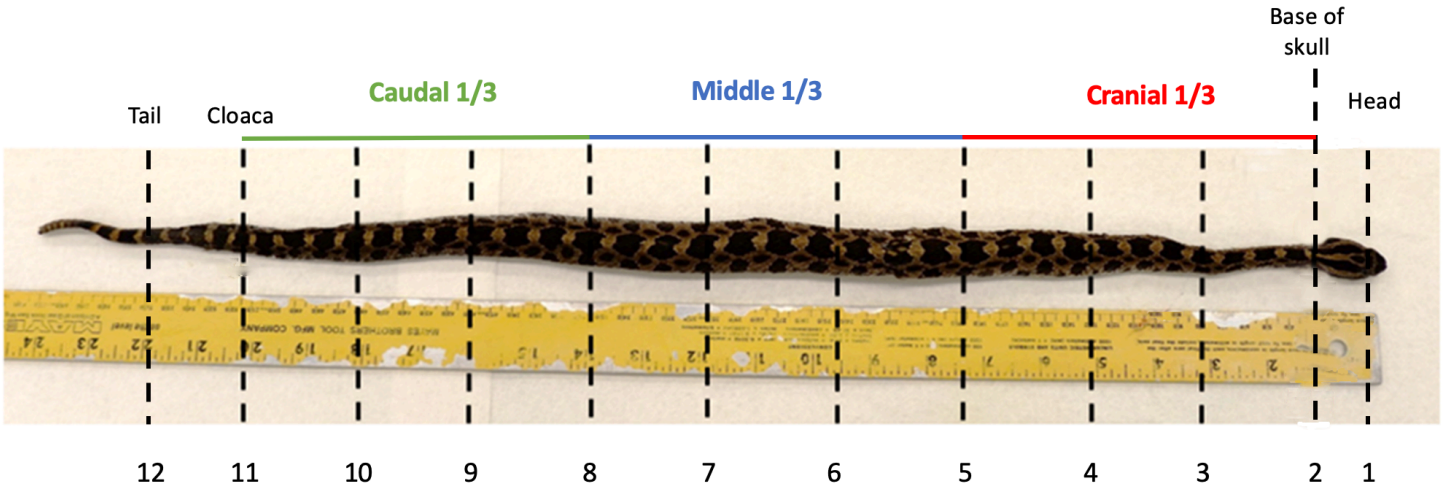

Figure 2.2. Adequate (A) and minimal (B) visceral adipose tissue surrounding intestines in Lake Erie watersnakes (*Nerodia sipedon insularum*).

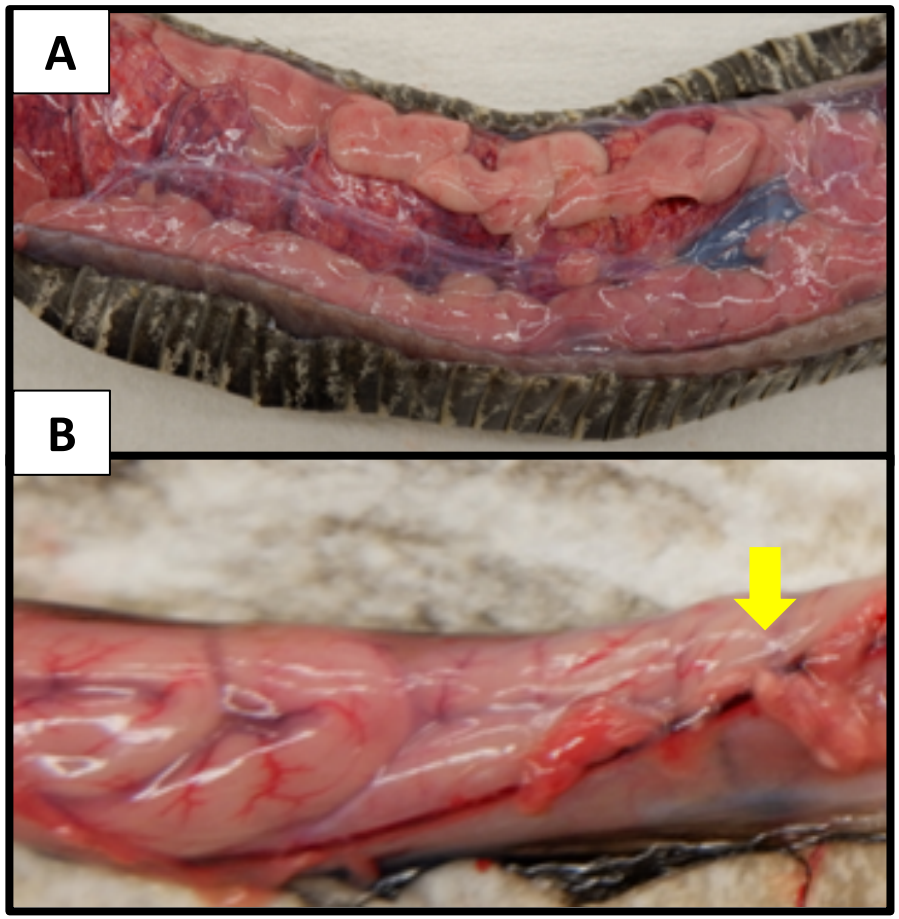

Figure 2.3. Full-thickness, circumferential section of a body survey section to be evaluated histologically.

Figure 2.4. Gross lesions associated with SFD in Lake Erie watersnakes (*Nerodia sipedone insularum*). A) Grade 1 cutaneous severity: heterophilic inflammation limited to the epidermis (arrows), mild hyperkeratosis (*), B) Grade 2 cutaneous severity: mixed inflammation extending into the dermis (arrow), mild hyperkeratosis (arrow head), extensive serocellular crusting (*). C) Grade 2 cutaneous severity: granulomatous inflammation limited to the dermis, discrete granuloma (*). D) Grade 2 cutaneous severity: granulomatous and heterophilic inflammation with multiple discrete granulomas (*). E) Grade 2 cutaneous severity: heterophilic epidermitis with dermal granuloma formation. F) Cross section of tail, Grade 3 cutaneous severity: epidermal ulceration and fungal crusts with coagulative necrosis of the epidermis, dermis, subcutis, and skeletal muscle. G) Longitudinal section of rostral maxilla, Grade 3 cutaneous severity: coagulative necrosis of the rostrum effacing regions of bone (box). H) Higher magnification of "G" showing bony changes: irregularly scalloped cancellous bone with numerous osteoblasts and fewer osteoclasts (arrows). I) Grade 3 cutaneous severity: heterophilic and granulomatous inflammation within the bone marrow space.

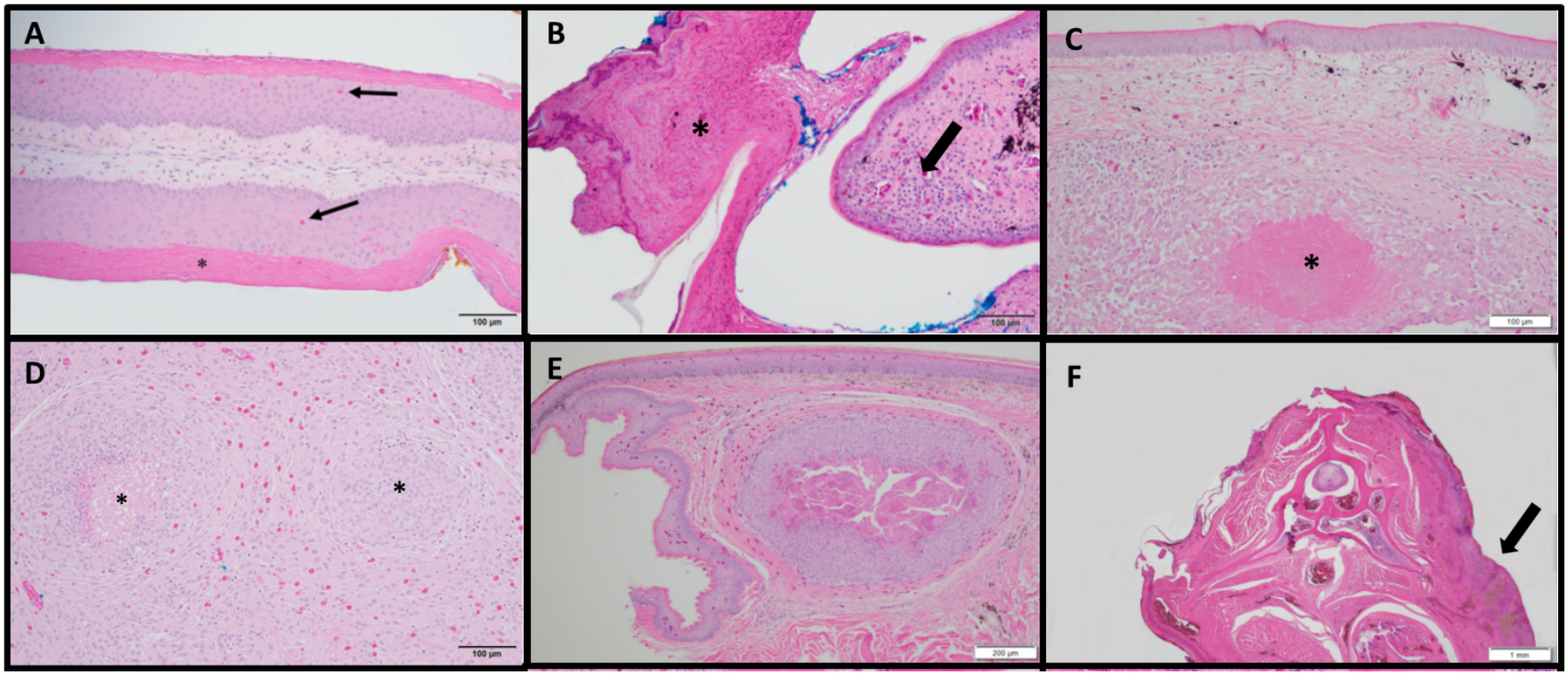

Figure 2.4 (continued).

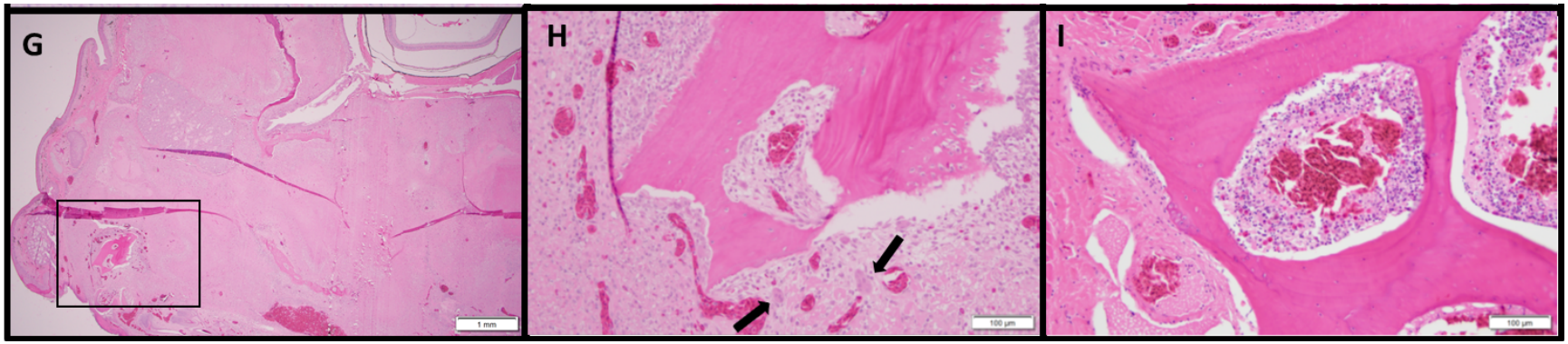

Figure 2.5. Gross lesions associated with SFD in Lake Erie watersnakes (*Nerodia sipedon insularum*). A) Multifocal crusts, B) Cluster of raised scales, C) Thickened scales (yellow arrow), D) Focal ulceration, E) Necrosis, F) Maxillary granuloma causing facial distortion.

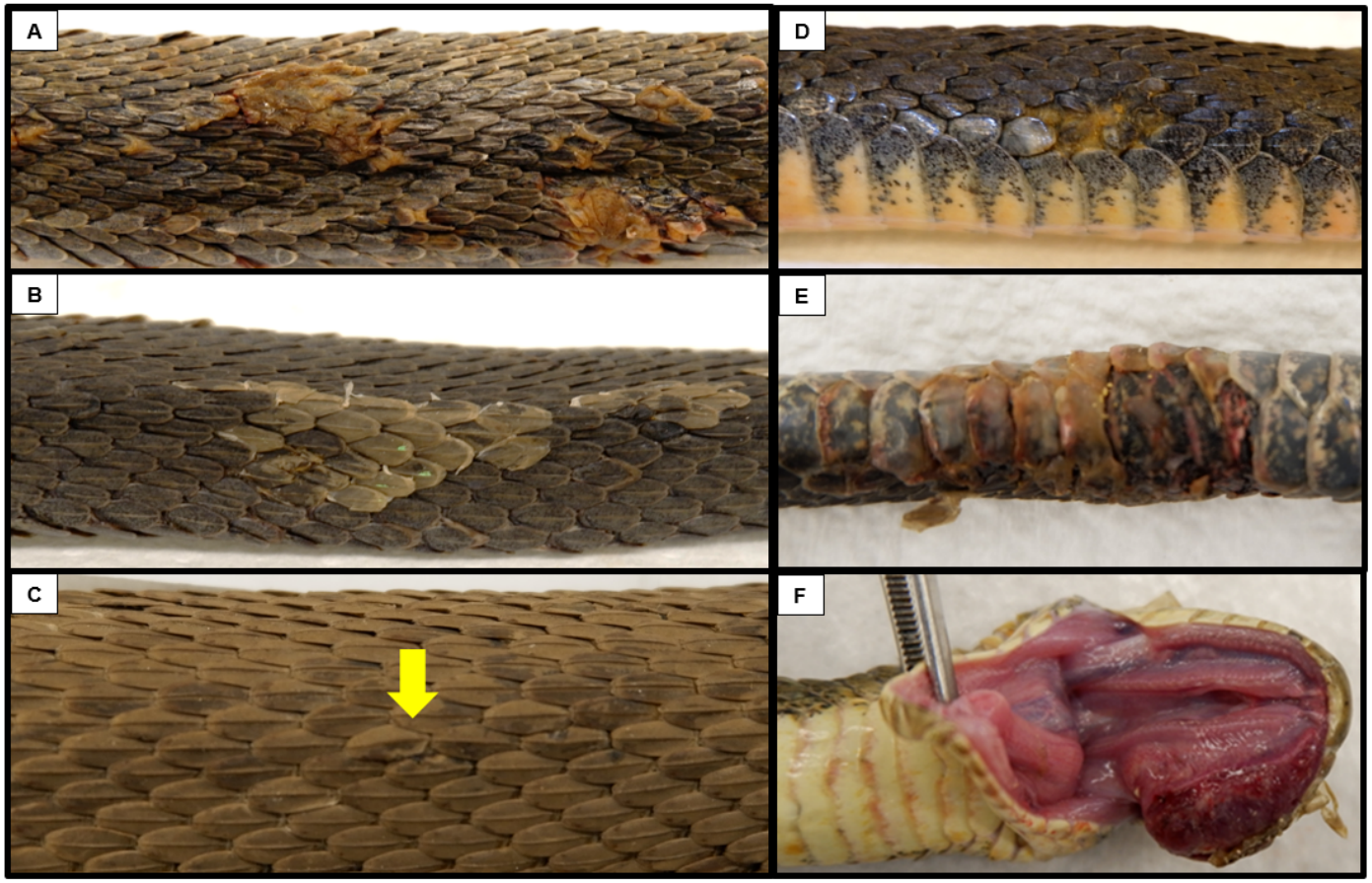

Table 2.1. Summary of demographic variables for 23 Lake Erie water snakes (*Nerodia sipedon insularum*).

ID	Sex	Age Class	Weight (g)	SVL (cm)[1]	Died/Euthanized	# of Treatment Months[2]	SFD Ante/Post-Mortem PCR	Days in Captivity
18-132	Female	Adult	322.53	84.5	Died	0	+	9
18-133	Female	Adult	371.42	91	Died	0	+	0
18-134	Female	Adult	298.00	86	Died	0	+	0
18-135	Male	Adult	149.14	72	Died	0	+	0
18-136	Female	Adult	285.56	84	Euthanized	0	+	0
18-137	Male	Adult	119.24	62	Euthanized	0	+	21
18-146	Male	Adult	92.11	59.5	Died	0	+	35
18-147	Male	Adult	99.74	61	Died	0	+	32
18-148	Male	Adult	183.79	74	Died	0	+	31
18-158	Female	Adult	255.00	93.5	Died	0.5	+	50
19-10	Male	Adult	157.88	72.5	Died	Saline Control	+	79
19-11	Female	Adult	270.46	85.5	Died	Saline Control	+	69
19-12	Female	Adult	220.94	81.5	Died	Saline Control	+	60
19-13	Female	Adult	290.50	88.5	Died	1	+	69
19-14	Male	Adult	130.00	67	Died	1.5	+	74
19-15	Male	Adult	139.06	71	Euthanized	1.5	+	135
19-16	Male	Adult	161.58	68	Died	2	+	106
19-17	Female	Adult	303.81	92	Died	Saline Control	+	104

[1] SVL indicated snout to vent (cloaca) length
[2] # of treatment months indicates total number of months the individual received terbinafine nebulization treatment.

Table 2.1 (continued)

ID	Sex	Age Class	Weight (g)	SVL (cm)	Died/Euthanized	# of Treatment Months	SFD Ante/Post-Mortem PCR	Days in Captivity
19-18	Female	Adult	278.97	87	Euthanized	Saline Control	+	162
19-19	Female	Adult	258.69	84	Euthanized	3	+	160
19-20	Female	Adult	351.95	90	Died	3	+	166
19-21	Female	Adult	291.85	88	Euthanized	2	+	110
19-22	Female	Adult	208.60	87	Euthanized	Saline Control	+	208

Table 2.2. Ophidiomycosis classification scheme according to Baker et al. (2019).

Classification	*O. ophiodiicola* qPCR/Culture results	Gross Lesions consistent with Ophidiomycosis	Histologic Lesions consistent with Ophidiomycosis
Ophidiomyces Present	+	-	-
Possible Ophidiomycosis	-	+	+
Apparent Ophidiomycosis	+	+	+ (hyphae, but NO arthroconidia)
Confirmed Ophidiomycosis	+	+	+ (arthroconidia present)

Table 2.3. Histopathologic grading scheme for histologic cutaneous lesions according to McKenzie et al. (2019). Each lesion is assigned to a histologic grade based on the total number of points. Grades are as follows: Grade 0 (0 total points), Grade 1 (0-3 total points), Grade 2 (4-5 total points), Grade 3 (6-8 total points).

Feature	Description	Points
Inflammation	None	0
	Heterophils in epidermis	1
	Mixed inflammation, dermal involvement	2
	Mixed inflammation, muscle involvement	3
Necrosis	None	0
	Minimal	1
	Coalescing areas, < 1 scale width	2
	Coalescing areas, > 1 scale width	3
Other	Serocellular crust	1
	Ulceration	1

Table 2.4. Presence of gross lesions in Lake Erie watersnakes (*Nerodia sipedon insularum*) within each individual and each survey section.

Snake	Head	1	2	3	4	5	6	7	8	9	Cloaca	Tail
18132	Crust	Raised scale, crust	None	Raised scale	None	Crust	Raised scale	None	None	None	None	Necrotic
18133	Thick scale	None	None	Ulcer	Thick scales	Crust	None	None	None	None	None	None
18134	Crust	Crust	Crust	Crust	Crust	Crust	Crust	Crust	Crust	Crust	Crust	Crust
18135	Thick scale	None	None	None	None	None	None	Raised scale, crust	None	None	None	None
18136	Thick scale, crust	None	Crust	None	None	None	Crust	None	None	None	None	None
18137	Crust, Granuloma	None	None	None	Raised scale	None	None	None	None	None	None	None
18146	Thick scale, crust	Crust	None	Crust	Raised scale	Raised scale	None	None	None	None	None	None
18147	Thick scale	None	None	None	None	Raised scale	None	None	Raised scale	None	None	Crust
18148	None	None	Thick scale	Thick scale	None	None	None	None	Thick scale	None	None	Necrosis
18158	Thick scale	None	None	None	None	None	None	None	None	None	None	Thick
1910	Raised, crusted scale	None	Raised scale	None	Crust	Crust	None	Crust	Crust	None	None	Thick
1911	Crust	Crust	Crust	Crust	Crust	Crust	Crust	Crust	Crust	None	None	Crust
1912	Crust	None	None	None	None	None	Crust	Crust	Crust	None	None	None
1913	None	None	None	Thick scale	None	Thick scale	None	None	None	None	None	None
1914	Crust	Raised scale	None	None	Raised scale	Raised scale	Raised scale	Raised scale	Raised scale	Raised scale	None	Raised scale
1915	Crust	Crust	None	Thick scale	None	Thick scale	None	None	None	None	None	Crust
1916	Crust	Crust	None	None	Raised scale	None	None	None	None	Raised scale	None	None
1917	Crust	Crust	Crust	Crust	Crust	Crust	Crust	None	Crust	Crust	None	Crust
1918	Crust	Crust	Crust	Crust	Crust	None	Crust	Crust	Crust	Crust	None	Crust
1919	Crust	Crust	None	None	None	None	None	None	Thick, raised scale	Crust	None	Crust
1920	Thick scale	Raised scale	None	None	None	Crust	Crust	Crust	None	None	None	None
1921	Crust	Crust	Crust	Crust	Crust	None	Crust	None	Raised scale	Raised scale	None	Raised scale
1922	None	Crust	Crust	Crust	None	None	None	Crust	None	Crust	Crust	Crust

Table 2.5. Prevalence of each type of gross lesion present in twelve survey sections from 23 Lake Erie watersnakes (*Nerodia sipedon insularum*).

	Crust	Raised Scale	Thickened Scale	Ulcer	Granuloma	Necrosis
Head	15 (65.2%)	0	7 (30.4%)	0	1 (4.3%)	0
1	11 (47.8%)	3 (13%)	0	0	0	0
2	7 (30.4%)	1 (4.3%)	1 (4.3%)	0	0	0
3	7 (30.4%)	1 (4.3%)	3 (13%)	1 (4.3%)	0	0
4	6 (26.1%)	4 (17.4%)	1 (4.3%)	0	0	0
5	7 (30.4%)	3 (13%)	2 (8.7%)	0	0	0
6	8 (34.8%)	2 (8.7%)	0	0	0	0
7	8 (34.8%)	2 (8.7%)	0	0	0	0
8	6 (26.1%)	4 (17.4%)	1 (4.3%)	0	0	0
9	5 (21.7%)	3 (13%)	0	0	0	0
Cloaca	2 (8.7%)	0	0	0	0	0
Tail	8 (34.8%)	3 (13%)	2 (8.7%)	0	0	2 (8.7%)

Table 2.6. Histologic cutaneous severity score of each survey section for each individual Lake Erei watersnake (*Nerodia sipedon insularum*).

Snake	Head	1	2	3	4	5	6	7	8	9	Cloaca	Tail
18132	3	2	2	2	2	3	2	3	1	0	0	3
18133	3	1	1	3	1	1	2	0	1	1	1	1
18134	3	3	3	3	2	2	3	2	2	3	1	1
18135	3	1	1	1	1	1	1	1	1	1	1	3
18136	3	1	1	1	1	1	1	1	1	1	2	0
18137	3	1	1	1	1	1	1	1	1	0	1	1
18146	3	3	1	2	2	1	1	1	1	2	3	3
18147	2	2	1	2	1	2	3	2	2	0	1	2
18148	2	2	1	2	1	1	1	1	1	1	3	3
18158	2	1	1	1	1	1	1	0	0	1	0	0
199	0	0	0	1	0	0	0	0	1	1	1	2
1910	2	1	2	2	2	1	2	1	2	2	1	2
1911	3	3	3	3	2	2	3	2	2	2	3	3
1912	2	1	1	1	1	1	2	1	2	1	1	1
1913	1	1	1	1	1	1	0	0	1	1	1	1
1914	3	2	1	1	2	2	1	2	2	2	2	2
1915	3	1	1	0	1	0	1	1	1	0	1	2
1916	3	1	0	0	1	1	1	0	1	1	2	0
1917	3	2	0	2	1	2	2	2	1	1	3	2
1918	1	2	2	1	2	2	1	1	1	1	1	1
1919	2	1	1	1	1	1	1	0	2	1	1	3
1920	3	1	1	1	1	1	1	1	1	1	1	1
1921	2	2	1	2	1	1	1	1	2	2	2	2
1922	1	1	1	2	1	1	1	1	1	1	1	2

Table 2.7. Prevalence of histologic lesions of SFD within 12 survey sections from 23 Lake Erie watersnakes (*Nerodia sipedon insularum*).

Survey Section	Number of snakes with histologic lesion in each section
Head	23 (100%)
1	23 (100%)
2	21 (91.3%)
3	21 (91.3%)
4	23 (100%)
5	22 (95.7%)
6	22 (95.7%)
7	18 (78.3%)
8	22 (95.7%)
9	19 (82.6%)
Cloaca	21 (91.3%)
Tail	20 (87%)
Cranial 1/3	23 (100%)
Middle 1/3	23 (100%)
Caudal 1/3	23 (100%)

Table 2.8. Median histologic severity score of each survey section from 23 Lake Erie watersnakes (*Nerodia sipedon insularum*).

Body Area	Median Histologic Severity Score	Minimum Histologic Severity Score	Maximum Histologic Severity Score
Head	3	0	3
1	1	0	3
2	1	0	3
3	1	0	3
4	1	0	2
5	1	0	3
6	1	0	3
7	1	0	3
8	1	0	2
9	1	0	3
Cloaca	1	0	3
Tail	2	0	3

Table 2.9. Prevalence of SFD-associated lesions (# of positive sections) and histological features in 23 Lake Erie watersnakes (*Nerodia sipedon insularum*).

Snake	# of Positive Sections	Sum of Severity Scores	Arthroconidia Present?
18132	10	23	Yes
18133	11	16	Yes
18134	12	28	Yes
18135	12	16	Yes
18136	11	14	Yes
18137	11	13	Yes
18146	12	23	Yes
18147	11	20	Yes
18148	12	19	Yes
18158	8	9	Yes
1910	12	20	Yes
1911	12	31	Yes
1912	12	15	Yes
1913	10	10	Yes
1914	12	22	Yes
1915	9	12	No
1916	8	11	No
1917	11	21	Yes
1918	12	16	Yes
1919	11	15	No
1920	12	14	Yes
1921	12	19	Yes
1922	12	14	Yes

Table 2.10. Pearson's correlation calculated between total number of sections with SFD-associated lesions and overall cutaneous histologic severity grade from 23 Lake Erie watersnakes (*Nerodia sipedon insularum*).

Survey Location	Correlation	P value
Head	0.333	0.120
1	0.869	0.000
2	0.643	0.001
3	0.742	0.000
4	0.712	0.000
5	0.643	0.001
6	0.713	0.000
7	0.744	0.000
8	0.570	0.005
9	0.548	0.007
Cloaca	0.435	0.038
Tail	0.554	0.006

Table 2.11. Number of survey sections within each cutaneous severity grade category that had evidence of subclinical vs. cutaneous gross lesions.

Histologic Grade	No Lesions. (# of sections)	Subclinical Lesions (# of sections)	Cutaneous Gross Lesions (# of sections)
0	18 (85.7%)	0 (0%)	3 (14.3%)
1	0 (0%)	98 (67.1%)	48 (32.9%)
2	0 (0%)	17 (23.6%)	55 (76.4%)
3	0 (0%)	2 (5.4%)	35 (94.6%)

Table 2.12. Comorbidities observed within the 23 Lake Erie watersnakes (*Nerodia sipedon insularum*).

Central Nervous System	Heart	Lungs	Liver	Splenopancreas	Gastrointestinal system	Kidneys	Subcutis	Skin	Visceral Adipose
heterophilic encephalitis (4.3%)	Vascular basement membrane mineralization (4.3%)	Nematodes (61%) Heterophilic interstitial pneumonia (4.3%) Granuloma (21.7%) Pulmonary fibrosis (4.3%)	Vascular basement membrane mineralization (4.3%) Fibrinonecrotizing hepatitis (8.7%) Granuloma (17.4%) Heterophilic hepatitis (4.3%) Vacuolar change (13%)	Granuloma (8.7%) Fibrinonecrotizing pancreatitis (4.3%) Heterophilic splenitis (17.4%) Multifocal necrosis (4.3%) Amyloidosis (4.3%)	Vascular basement membrane mineralization (4.3%) Nematodes (39%) Fibrinonecrotizing and hemorrhagic peritonitis (4.3%) Heterophilic gastritis (4.3%) Mesenteric granuloma (4.3%) Heterophilic enteritis (4.3%) Ciliated protozoa (4.3%) Coccidia (13%) Heterophilic colitis (4.3%) Lymphoplasmacytic colitis (4.3%)	Vascular basement membrane mineralization (4.3%) Intratubular mineral (8.7%) Granuloma (4.3%) Lymphoplasmacytic interstitial nephritis (4.3%) Multifocal tubular necrosis with renal gout (4.3%)	Granuloma (13%)	Bacteria (100%) Mites (21.7%) Algae (4.3%)	decreased visceral adipose tissue stores (82.6%)

Table 2.13. All comorbidities observed in 23 Lake Erie watersnakes (*Nerodia sipedon insularum*). NSL= no significant lesions.

Snake	Liver	Heart	Lungs	Splenopancreas	Stomach	Small intestine	Subcutis	Kidneys	Large intestine	Skin	Other	Brain
18132	Mineralization of basement membranes	Mineralization of basement membranes	Nematodes	Granulomas	Nematodes, mineralization of basement membranes	Mineralization of basement membranes	NSL	Granulomas, mineralization of basement membranes	Mineralization of basement membranes	Algae & bacteria	NSL	NSL
18133	Fibrinonecrotizing hepatitis with bacteria	NSL	Mild heterophilic interstitial pneumonia	NSL	Nematodes, fibrinonecrotic & hemorrhagic peritonitis	Nematodes, fibrinonecrotic & hemorrhagic peritonitis	NSL	NSL	Nematodes, fibrinonecrotic & hemorrhagic peritonitis	Bacteria	Severe thoracic ulceration	NSL
18134	NSL	NSL	NSL	NSL	NSL	Intraluminal nematodes	NSL	NSL	NSL	Bacteria	NSL	NSL
18135	Granulomas & Fibrinonecrotizing hepatitis	NSL	NSL	Mild Fibrinonecrotizing pancreatitis	NSL	Ciliated protozoa	NSL	NSL	Ciliated protozoa	Bacteria	NSL	NSL
18136	NSL	NSL	NSL	NSL	Mild heterophilic gastritis	Mild heterophilic enteritis	Granulomas with fungus	NSL	Moderate heterophilic colitis	Bacteria and mites	NSL	NSL
18137	Mild heterophilic hepatitis & multifocal necrosis	NSL	Granulomas, pulmonary fibrosis, mild bronchiolar hyperplasia	Mild heterophilic splenitis, multifocal necrosis	Mesenteric granuloma	NSL	NSL	NSL	NSL	Bacteria	NSL	NSL
18146	NSL	NSL	Nematodes	NSL	Nematode	Intraepithelial Coccidia	NSL	NSL	NSL	Bacteria	Tracheal granulomas	NSL
18147	NSL	NSL	Nematodes	NSL	NSL	NSL	NSL	Intratubular mineral	Intraluminal nematodes	Bacteria	NSL	Moderate heterophilic encephalitis
18148	NSL	NSL	NSL	Mild heterophilic splenitis	NSL	NSL	Granuloma	NSL	NSL	Bacteria	NSL	NSL
18158	Moderate amyloidosis	NSL	Nematodes	Moderate amyloidosis	NSL	Intraluminal nematodes	NSL	Mild lymphoplasmacytic interstitial nephritis	Intraluminal nematodes	Bacteria	NSL	NSL
199	NSL	NSL	Nematodes	NSL	NSL	Nematodes	NSL	NSL	Nematodes	Bacteria	NSL	NSL
1910	Mild vacuolar change	NSL	Nematodes	NSL	Nematodes	Intraluminal coccidia	NSL	NSL	NSL	Bacteria	Focal granulomatous stomatitis, osteomyelitis	NSL
1911	NSL	NSL	Nematodes	NSL	NSL	NSL	NSL	NSL	NSL	Bacteria	NSL	NSL
1912	NSL	NSL	Granulomas	NSL	NSL	Coccidia	NSL	NSL	Coccidia	Bacteria	NSL	NSL
1913	NSL	NSL	Nematodes	NSL	NSL	NSL	NSL	NSL	NSL	Bacteria	NSL	NSL
1914	NSL	NSL	Nematodes	NSL	NSL	NSL	NSL	Intratubular mineral	NSL	Bacteria & mites	Thoracic granuloma	NSL
1915	Granuloma	NSL	Granuloma, nematodes	Granulomas	NSL	NSL	Granulomas	Granulomas	NSL	Bacteria	Head: osteomyelitis with bacteria; tracheal granuloma	NSL

Table 2.13 (continued)

Snake	Liver	Heart	Lungs	Splenopancreas	Stomach	Small intestine	Subcutis	Kidneys	Large intestine	Skin	Other	Brain
1916	Granuloma	NSL	Granuloma	NSL	NSL	NSL	NSL	NSL	NSL	Bacteria	NSL	NSL
1917	NSL	NSL	Nematodes	NSL	NSL	NSL	NSL	NSL	NSL	Bacteria	NSL	NSL
1918	Granuloma	NSL	Granuloma	NSL	NSL	NSL	NSL	NSL	NSL	Bacteria	NSL	NSL
1919	NSL	NSL	NSL	NSL	NSL	NSL	NSL	NSL	NSL	Bacteria	NSL	NSL
1920	Mild vacuolar change	NSL	Nematodes	Mild heterophilic splenitis	NSL	NSL	NSL	Multifocal tubular necrosis with renal gout	NSL	Bacteria & mites	Histiocytic and necrotizing stomatitis and osteomyelitis	NSL
1921	NSL	NSL	Nematodes	NSL	NSL	Nematodes	NSL	NSL	Nematodes	Bacteria & mites	Oviduct: presumed leiomyoma	NSL
1922	Mild vacuolar change	NSL	Nematodes	Mild heterophilic splenitis	NSL	Moderate lymphoplasmacytic enteritis	NSL	NSL	Moderate lymphoplasmacytic colitis	Bacteria & mites	NSL	NSL

CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

The goal of this project was to characterize the pathology of ophidiomycosis (snake fungal disease, SFD), an emerging infectious disease that is the cause of individual and population mortality events. The study focused on the pathology of SFD in Lake Erie watersnakes. In the first hypothesis, the distribution and prevalence of gross and histologic disease was documented. The lesions identified in the first objective were characterized using a recently developed grading scheme that allowed objective documentation of cutaneous severity. With this data, it was found that as overall cutaneous severity of an individual increased, cutaneous lesions tended to be more widely distributed. This study also demonstrated that subclinical disease was present in 45.3% of survey sections throughout this cohort of snakes. The high prevalence of subclinical disease in this group highlights the need for pathologic assessment in addition to molecular/microbiological testing to confirm SFD and demonstrates that solely relying on qPCR may underestimate disease prevalence.

Future study should be aimed at detecting *O. ophiodiicola* in characteristic subclinical cutaneous lesions. During this study, cutaneous swabs were taken at each of the survey locations (head, tail, cloaca, and nine body sections), but qPCR testing of these swabs was beyond the scope of this project. Detecting *O. ophiodiicola* in these lesions would establish a direct link that can be applied to characterize disease progression. The preferred approach to characterize these lesions would be an experimental challenge study. Ideally, a cohort of untreated, naïve snakes could be inoculated in multiple areas of the body with *O. ophiodiicola* either simply on the epidermal surface or facilitated by a break in the epidermis. Skin biopsies could then be taken sequentially in order to document the temporal changes associated with each phase of the disease

process. Differences in success of individuals to resolve characteristic lesions could then be used to identify early less invasive diagnostic tests or treatment options that improve success.

Results of this study showed that cutaneous severity did not correlate with the presence or absence of visceral granulomas. One of the major limitations of this study was that visceral granulomas were not confirmed to be directly associated with *O. ophiodiicola* infection. If these granulomas truly represent systemic infection by *O. ophiodiicola*, this would provide additional documentation with regard to the prevalence of true internal lesions, which is rarely reported in the literature. Confirming that these granulomas are directly caused by *O. ophiodiicola* would also add further support to our conclusion that multiple modalities of testing are needed to confirm SFD, as a cutaneous swab would not be adequate to rule out SFD in these cases. Future work should be directed at qPCR of these visceral granulomas for *O. ophiodiicola*, *Mycobacteria sp.,* and other bacteria such as *Morganella sp.*, *Salmonella sp.*, and *Chlamydophila sp.* to rule out a coinfection. Definitively diagnosing the underlying cause of these visceral granulomas is pertinent to understanding the pathogenesis of systemic infection and/or understanding how systemic granuloma formation is capable of contributing to mortality.

The results of this study demonstrated numerous comorbidities within this sample population. Histologic characterization of these findings suggested that many common comorbidities (e.g. pulmonary and gastrointestinal nematodes) were likely incidental and did not significantly contribute to the animal's death. Based on these findings, it was concluded that SFD alone is capable of causing individual mortality. However, the mechanism by which it does so remains unclear. This thesis showed that the most prevalent comorbidity was a marked reduction in visceral adipose. This finding may indicate a persistent negative energy balance secondary to SFD that could explain how *O. ophiodiicola* infection alone could lead to death. Future studies

should include wider assessment of body condition, including quantification of muscle mass. A future challenge study could use 3 groups of snakes offered small, moderate, and high caloric intake. These individuals could then be assessed for body condition by measuring abdominal girth measurement, muscle mass, and body weight. This could be investigated by using computed tomography or the use of metabolomic methods. Results may also provide insight as to whether or not available nutrition is associated with resolution of SFD.

This study was focused on characterization of SFD specifically in Lake Erie watersnakes. A pertinent future direction would include repeating this same protocol on snakes of varying species and natural habitats in order to see if the results stated in Chapter 2 hold true. During this study period, 13 additional snakes, including three Eastern Massasauga rattlesnakes (*Sistrurus catenatus*), four prairie kingsnakes (*Lampropeltis calligaster*), two blue racers (*Coluber constrictor foxii*), one black rat snake (*Pantherophis obsoletus*), one yellow rat snake (*Pantherophis alleghaniensis*), one timber rattlesnake (*Crotalus horridus*), and one Eastern fox snake (*Pantherophis gloydi*) were examined using this study's protocol; however, because of the variability in species, natural habitat, length in captivity, these individuals were not included in the data set. Continued recruitment of snakes of various species such as the ones previously listed, particularly if variables can be controlled, may offer insight into how the disease may manifest in different species and environments.

This thesis provides a basis for future consideration in studies evaluating prevalence of SFD in individual snakes or snake populations by providing a standardized approach to gross and histopathologic examination as well as highlighting the high prevalence of subclinical disease and necessity of using fungal culture/PCR, gross findings, and histopathology in documentation of disease. By documenting a high prevalence of subclinical disease, this study also informs

those tasked with providing effective treatment to SFD affected individuals that a lack of, or resolution of, gross lesions does not imply resolution of disease. The work done in this study provides a starting point for veterinary pathologists, clinicians, and researchers alike to collect appropriate samples to accurately identify and document SFD in both treated and untreated snakes.

REFERENCES

Agugliaro, J., Lind, C.M., Lorch, J.M. & Farrell, T.M. (2019). An emerging fungal pathogen is associated with increased restring metabolic rate and total evaporative water loss rate in a winter-active snake. *Functional Ecology*, 34, 486-496.

Allender, M. C., Baker, S., Britton, M., & Kent, A. D. (2018). Snake fungal disease alters skin bacterial and fungal diversity in an endangered rattlesnake. *Scientific Reports*, *8*(1), 1–9. doi:10.1038/s41598-018-30709-x

Allender, M. C., Baker, S., Wylie, D., Loper, D., Dreslik, M. J., Phillips, C. A., Maddox, C., & Driskell, E. A. (2015a). Development of snake fungal disease after experimental challenge with *Ophidiomyces ophiodiicola* in cottonmouths (*Agkistrodon piscivorous*). *PLoS One*, *10*(10), e0140193. doi:10.1371/journal.pone.0140193

Allender, M. C., Bunick, D., Dzhaman, E., Burrus, L., & Maddox, C. (2015b). Development and use of a real-time polymerase chain reaction assay for the detection of *Ophidiomyces ophiodiicola* in snakes. *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, *27*(2), 217–220. doi:10.1177/1040638715573983

Allender, M. C., Hileman, E. T., Moore, J., & Tetzlaff, S. (2016a). Detection of *Ophidiomyces*, the causative agent of snake fungal disease, in the Eastern Massasauga (*Sistrurus catenatus*) in Michigan, USA, 2014. *Journal of Wildlife Diseases*, *52*(3), 694–698. doi:10.7589/2015-12-333

Allender, M. C., Junge, R. E., Baker-Wylie, S., Hileman, E. T., Faust, L. J., & Cray, C. (2015c). Plasma electrophoretic profiles in the Eastern Massasauga (*Sistrurus catenatus*) and influences of age, sex, year, location, and snake fungal disease. *Journal of Zoo and Wildlife*

Medicine: Official Publication of the American Association of Zoo Veterinarians, *46*(4), 767–773. doi:10.1638/2015-0034.1

Allender, M. C., Phillips, C. A., Baker, S. J., Wylie, D. B., Narotsky, A., & Dreslik, M. J. (2016b). Hematology in an Eastern Massasauga (*Sistrurus catenatus*) population and the emergence of *Ophidiomyces* in Illinois, USA. *Journal of Wildlife Diseases*, *52*(2), 258–269. doi:10.7589/2015-02-049

Allender, M. C., Raudabaugh, D. B., Gleason, F. H., & Miller, A. N. (2015d). The natural history, ecology, and epidemiology of *Ophidiomyces ophiodiicola* and its potential impact on free-ranging snake populations. *Fungal Ecology*, *17*(October 2015), 187–196.

Allender, M.C., Dreslik, M., Wylie, S., Phillips, C., Wylie, D.B., Maddox, C., Delaney, M.A., Kinsel, M.J. (2011). *Chrysosporium sp.* infection in Eastern Massasauga rattlesnakes. *Emerging Infectious Diseases,* 17, 2383-2384.

Baker, S. J., Haynes, E., Gramhofer, M., Stanford, K., Bailey, S., Christman, M., Conley, K., Frasca, S. Jr., Ossiboff, R. J., LoBato, D., & Allender, M. C. (2019). Case definition and diagnostic testing for snake fungal disease. *Herpetological Review*, *50*(2), 279–285.

Barber, D., Poole, V., Sanchez, C., Roady, P., & Allender, M. (2016). Snake fungal infection associated with *Fusarium* found in *Nerodia erythrogaster transversa* (Blotched Water Snake) in Texas, USA. *Herpetological Review*, *47*, 39–42.

Beisel, W.R. (1975). Metabolic response to infection. *Annual Review of Medicine*, 26, 9-20. doi: 10.1146/annurev.me.26.020175.000301

Bohuski, E., Lorch, J. M., Griffin, K. M., & Blehert, D. S. (2015). TaqMan real-time polymerase chain reaction for detection of *Ophidiomyces ophiodiicola*, the fungus associated with snake fungal disease. *BMC Veterinary Research*, *11*, 95. doi:10.1186/s12917-015-0407-8

Burbrink, F. T., Lorch, J. M., & Lips, K. R. (2017). Host susceptibility to snake fungal disease is highly dispersed across phylogenetic and functional trait space. *Science Advances*, *3*(12), e1701387. doi:10.1126/sciadv.1701387

Burnham, D.K., Keall, S.N., Nelson, N.J., & Daugherty, C.H. (2005). T cell function in tuatara (*Sphenodon punctatus)*. *Comp. Immunol. Microbiol. Infec. Dis.* 28(3), 213-222.

Casadevall, A., Steenbergen, J. N., & Nosanchuk, J. D. (2003). "Ready made" virulence and "dual use" virulence factors in pathogenic environmental fungi—the *Cryptococcus neoformans* paradigm. *Current Opinion in Microbiology*, *6*(4), 332–337. doi:10.1016/s1369-5274(03)00082-1

Chandler, H. C., Allender, M. C., Stegenga, B. S., Haynes, E., Ospina, E., & Stevenson, D. J. (2019). Ophidiomycosis prevalence in Georgia's Eastern Indigo Snake (*Drymarchon couperi*) populations. *PLoS One*, *14*(6). doi:10.1371/journal.pone.0218351

Clark, R.W., Marchand, M.N., Clifford, B.J., Stechert, R., Stephens, S. (2011). Decline of an isolated timber rattlesnake (*Crotalus horridus*) population: Interactions between climate change, disease, and loss of genetic diversity. *Biological Conservation*, 144, 886-891.

Department of Defense. Legacy Resource Management Program. (2019). *DoD snake fungal disease survey: natural resource manager training and data collection* (Report No. 17-838). Retrieved from https://www.denix.osd.mil/dodparc/parc-resources/dod-parc-reports/final-report-legacy-project-17-838-dod-snake-fungal-disease-survey-natural-resources-manager-training-and-data-collection/

Dolinski, A. C., Allender, M. C., Hsiao, V., & Maddox, C. W. (2014). Systemic *Ophidiomyces ophiodiicola* infection in a free-ranging plains garter snake (*Thamnophis radix*). *Journal of Herpetological Medicine and Surgery*, *24*(1), 7–10. doi:10.5818/1529-9651-24.1.7

Fisher, M. C., Henk, Daniel. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., & Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature*, *484*(7393). doi:10.1038/nature10947

Franklinos, L. H. V., Lorch, J. M., Bohuski, E., Rodriguez-Ramos Fernandez, J., Wright, O. N., Fitzpatrick, L., Petrovan, S., Durrant, C., Linton, C., Baláž, V., Cunningham, A. A., & Lawson, B. (2017). Emerging fungal pathogen *Ophidiomyces ophiodiicola* in wild European snakes. *Scientific Reports*, *7*(1), 1–7. doi:10.1038/s41598-017-00352-1

Glorioso, B. M., Waddle, J. H., Green, D. E., & Lorch, J. M. (2016). First documented case of snake fungal disease in a free-ranging wild snake in Louisiana. *Southeastern Naturalist*, *15*(1), 46.

Grisnik, M., Leys, J. E., Bryan, D., Hardman, R., Miller, D., Cobb, V., Ogle, C., Simpson, C., Campbell, J., Applegate, R., Allender, M., Nordberg, E., Hoekstra, A., & Walker, D. M. (2018). Host and geographic range of snake fungal disease in Tennessee, USA. *Herpetological Review*, *49*(4), 682–690.

Guthrie, A. L., Knowles, S., Ballmann, A. E., & Lorch, J. M. (2016). Detection of snake fungal disease due to *Ophidiomyces ophiodiicola* in Virginia, USA. *Journal of Wildlife Diseases*, *52*(1), 143–149. doi:10.7589/2015-04-093.1

Hawley, D.M., DuRant, S.E., Wilson, A.F., Adelman, J.S., & Hopkins W.A. (2012). Additive metabolic costs of thermoregulation and pathogen infection. *Functional Ecology*, 26(3), 701-710.

Hileman, E. T., Allender, M. C., Bradke, D. R., Faust, L. J., Moore, J. A., Ravesi, M. J., & Tetzlaff, S. J. (2018). Estimation of *Ophidiomyces* prevalence to evaluate snake fungal

disease risk. *The Journal of Wildlife Management*, *82*(1), 173–181. doi:10.1002/jwmg.21345

Januszkiewicz, E., Chinnici, N., & Laduke, T. C. (2019). Detection of snake fungal disease caused by *Ophidiomyces ophiodiicola* among timber rattlesnakes (*Crotalus horridus*) in Pennsylvania, USA. *Herpetological Review*, *50*(2), 286–288.

Kane, L. P., Allender, M. C., Archer, G., Leister, K., Rzadkowska, M., Boers, K., Souza, M., & Cox, S. (2017). Pharmacokinetics of nebulized and subcutaneously implanted terbinafine in cottonmouths (*Agkistrodon piscivorus*). *Journal of Veterinary Pharmacology and Therapeutics*, *40*(5), 575–579. doi:10.1111/jvp.12406

Kerl, M.E. (2003). Update on canine and feline fungal diseases. *Veterinary Clinics of North America: Small Animal Practice*, 33(4), 721-747.

Last, L. A., Fenton, H., Gonyor-McGuire, J., Moore, M., & Yabsley, M. J. (2016). Snake fungal disease caused by *Ophidiomyces ophiodiicola* in a free-ranging mud snake (*Farancia abacura*). *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, *28*(6), 709–713. doi:10.1177/1040638716663250

Learn, J.R. (2019, November 7). California officials confirm snake fungal disease in state. Retrieved February 8, 2020 from https://wildlife.org/california-officials-confirm-snake-fungal-disease-in-state/

Licitra, D., Quinn, D. P., Reeder, J. E., Gavitt, T., Dickson, J., Hess, B., Mangold, B. J., Tuttle, A. D., Rosas-Rosas, A., Frasca, S., & Szczepanek, S. M. (2019). Snake fungal disease in colubridae snakes in Connecticut, USA in 2015 and 2017. *Journal of Wildlife Diseases*, *55*(3), 658–662. doi:10.7589/2018-04-100

Lillywhite, H. B., Menon, J. G., Menon, G. K., Sheehy, C. M., & Tu, M. C. (2009). Water exchange and permeability properties of the skin in three species of amphibious sea snakes (*Laticauda* spp.). *The Journal of Experimental Biology*, *212*(Pt 12), 1921–1929. doi:10.1242/jeb.028704

Lind, C.M., Clark, A., Smiley-Walters, S.A., Taylor, D.R., Isidoro-Ayza, M., Lorch, J.M., & Farrell, T.M. (2019a). Interactive effects of food supplementation and snake fungal disease on pregnant pygmy rattlesnakes and their offspring. *Journal of Herpetology*, 53(4), 282-288.

Lind, C. M., Lorch, J. M., Moore, I. T., Vernasco, B. J., & Farrell, T. M. (2019b). Seasonal sex steroids indicate reproductive costs associated with snake fungal disease. *Journal of Zoology*, *307*(2), 104–110. doi:10.1111/jzo.12628

Lind, C., Moore, I. T., Akçay, Ç., Vernasco, B. J., Lorch, J. M., & Farrell, T. M. (2018a). Patterns of circulating corticosterone in a population of rattlesnakes afflicted with snake fungal disease: stress hormones as a potential mediator of seasonal cycles in disease severity and outcomes. *Physiological and Biochemical Zoology: PBZ*, *91*(2), 765–775. doi:10.1086/695747

Lind, C. M., McCoy, C. M., & Farrell, T. M. (2018b). Tracking outcomes of snake fungal disease in free-ranging pygmy rattlesnakes (*Sistrurus miliarius*). *Journal of Wildlife Diseases*, *54*(2), 352–356. doi:10.7589/2017-05-109

Lindemann, D. M., Allender, M. C., Rzadkowska, M., Archer, G., Kane, L., Baitchman, E., Driskell, E. A., Chu, C. T., Singh, K., Hsiao, S.-H., Sykes, J. M., & Cox, S. (2017). Pharmacokinetics, efficacy, and safety of voriconazole and itraconazole in healthy cottonmouths (*Agkistrodon piscivorus*) and Massasauga rattlesnakes (*Sistrurus catenatus*)

with snake fungal disease. *Journal of Zoo and Wildlife Medicine: Official Publication of the American Association of Zoo Veterinarians*, *48*(3), 757–766. doi:10.1638/2016-0179.1

Long, R. B., Love, D., Seeley, K. E., Patel, S., Allender, M. C., Garner, M. M., & Ramer, J. (2019). Host factors and testing modality agreement associated with *Ophidiomyces* infection in a free-ranging snake population in southeast Ohio, USA. *Journal of Zoo and Wildlife Medicine: Official Publication of the American Association of Zoo Veterinarians*, *50*(2), 405–413. doi:10.1638/2018-0143

Lorch, J. M., Knowles, S., Lankton, J. S., Michell, K., Edwards, J. L., Kapfer, J. M., Staffen, R. A., Wild, E. R., Schmidt, K. Z., Ballmann, A. E., Blodgett, D., Farrell, T. M., Glorioso, B. M., Last, L. A., Price, S. J., Schuler, K. L., Smith, C. E., Wellehan, J. F. X., & Blehert, D. S. (2016). Snake fungal disease: An emerging threat to wild snakes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1709). doi:10.1098/rstb.2015.0457

Lorch, J. M., Lankton, J. S., Werner, K., Falendysz, E. A., McCurley, K., & Blehert, D. S. (2015). Experimental infection of snakes with *Ophidiomyces ophiodiicola* causes pathological changes that typify snake fungal disease. *MBio*, *6*(6). doi:10.1128/mBio.01534-15

McCoy, C. M., Lind, C. M., & Farrell, T. M. (2017). Environmental and physiological correlates of the severity of clinical signs of snake fungal disease in a population of pigmy rattlesnakes, *Sistrurus miliarius*. *Conservation Physiology*, *5*(1), cow077. doi:10.1093/conphys/cow077

McKenzie, J. M., Price, S. J., Fleckenstein, J. L., Drayer, A. N., Connette, G. M., Bohuski, E., & Lorch, J. M. (2019). Field diagnostics and seasonality of *Ophidiomyces ophiodiicola* in wild snake populations. *EcoHealth*, *16*(1), 141–150. doi:10.1007/s10393-018-1384-8

McKenzie, C., Jardine, C., Campbell, D., Stevens, B., Oesterle, P., Shirose, L., Davy, C., Lillie, B., Nemeth, N. (2019). Pathology in snakes naturally and experimentally infected with *Ophidiomyces ophiodiicola*. Paper presented at the 68th Annual Wildlife Disease Association International Conference Abstract, Tahoe City, California, USA. Retrieved from https://aut.ac.nz.libguides.com/APA6th/conferences

Ndahimana, D., & Kim, E. K. (2018). Energy requirements in critically ill patients. *Clinical Nutrition Research*, *7*(2), 81–90. https://doi.org/10.7762/cnr.2018.7.2.81

Natarajan, K. & Muthukkaruppan, V.R. (1985). Distribution and ontogeny of B cells in the garden lizard, *Calotes versicolor*. *Developmental and Comparative Immunology,* 9(2), 301-310.

Ohkura, M., Worley, J. J., Hughes-Hallett, J. E., Fisher, J. S., Love, B. C., Arnold, A. E., & Orbach, M. J. (2016). *Ophidiomyces ophiodiicola* on a captive black racer (*Coluber constrictor*) and a garter snake (*Thamnophis sirtalis*) in Pennsylvania. *Journal of Zoo and Wildlife Medicine: Official Publication of the American Association of Zoo Veterinarians*, *47*(1), 341–346. doi:10.1638/2015-0123.1

Origgi, F.C. (2007). Chapter 2: Reptile Immunology. In E.R. Jacobson (Ed.), *Infectious Disease and Pathology of Reptiles* (pp.131-151). Taylor & Francis Group.

Otálora-Ardila, A., Herrera M, L. G., Flores-Martínez, J. J., & Welch, K. C., Jr (2016). Metabolic cost of the activation of immune response in the fish-eating myotis (Myotis

vivesi): The effects of inflammation and the acute phase response. *PLoS One*, *11*(10), e0164938. https://doi.org/10.1371/journal.pone.0164938

Paré, J. A., & Sigler, L. (2016). An overview of reptile fungal pathogens in the genera *Nannizziopsis*, *Paranannizziopsis*, and *Ophidiomyces*. *Journal of Herpetological Medicine and Surgey*, *26*(1–2), 46–53.

Park, H., Jang, H., Kim, C., Chung, B., Chang,, C.L., Park, S.K., & Song, S. (2000). Detection and identification of mycobacteria by amplification of the internal transcribed spacer regions with genus- and species-specific PCR primers. *J Clin Microbiol,* 38(11): 4080-5.

Picquet, P., Heckers, K. O., Kolesnik, E., Heusinger, A., & Marschang, R. E. (2018). Detection of *Ophidiomyces ophiodiicola* in two captive bocourt water snakes (*Subsessor bocourti*) and one captive pueblan milk snake (*Lampropeltis triangulum campbelli*). *Journal of Zoo and Wildlife Medicine: Official Publication of the American Association of Zoo Veterinarians*, *49*(1), 219–222. doi:10.1638/2017-0112R.1

Rajeev, S., Sutton, D. A., Wickes, B. L., Miller, D. L., Giri, D., Meter, M. V., Thompson, E. H., Rinaldi, M. G., Romanelli, A. M., Cano, J. F., & Guarro, J. (2009). Isolation and characterization of a new fungal species, *Chrysosporium ophiodiicola*, from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*). *Journal of Clinical Microbiology*, *47*(4), 1264–1268. doi:10.1128/JCM.01751-08

Ravesi, M. J., Tetzlaff, S. J., Allender, M. C., & Kingsbury, B. A. (2016). Detection of snake fungal disease from a *Lampropeltis triangulum* (Eastern milksnake) in northern Michigan. *Northeastern Naturalist*, *23*(3). doi:10.1656/045.023.0310

Ridi, R. E., Badir, N., & Rouby, S. E. (1981). Effect of seasonal variations on the immune system of the snake, *Psammophis schokari*. *Journal of Experimental Zoology*, *216*(3), 357–365. doi:10.1002/jez.1402160303

Rzadkowska, M., Allender, M. C., O'Dell, M., & Maddox, C. (2016). Evaluation of common disinfectants effective against *Ophidiomyces ophiodiicola*, the causative agent of snake fungal disease. *Journal of Wildlife Diseases*, *52*(3), 759–762. doi:10.7589/2016-01-012

Sandmeier, F., Horn, K., Tracy, R. (2016). Temperature-independent, seasonal fluctuations in immune-function in a reptile, the Mojave desert tortoise (*Gopherus agassizii*). *Canadian Journal of Zoology*, 94(8), 583-590. doi:10.1139/cjz-2016-0010

Schaefer, A., Condrey, K., Hymes, J., Goetz, N., Jarchow, J., Thangamani, S., Struthers, J. (2019). *Ophidiomyces ophiodiicola* epizootic in a captive collection of snakes in Arizona. Paper presented at the 70th Annual American College of Veterinary Pathologists Conference Abstract, San Antonio, Texas, USA. Retrieved from https://cdn.ymaws.com/www.acvp.org/resource/resmgr/meetings_&_events/2019/2019_acvp_and_asvcp_final_ab.pdf

Shine, R., Goiran, C., Shilton, C., Meiri, S., & Brown, G. P. (2019). The life aquatic: An association between habitat type and skin thickness in snakes. *Biological Journal of the Linnean Society*, blz136. doi:10.1093/biolinnean/blz136

Sigler, L., Hambleton, S., & Paré, J. A. (2013). Molecular characterization of reptile pathogens currently known as members of the *Chrysosporium* Anamorph of *Nannizziopsis vriesii* complex and relationship with some human-associated isolates. *Journal of Clinical Microbiology*, *51*(10), 3338–3357. doi:10.1128%2FJCM.01465-13

Steeil, J. C., Hope, K. L., Evans, M., Peters, A., & Cartoceti, A. (2018). Multifocal *Ophidiomyces ophiodiicola* infection in an aastern diamondback rattlesnake (*Crotalus adamanteus*) without the presence of skin lesions. *Journal of Herpetological Medicine and Surgery*, *28*(3–4), 76–80. doi:10.5818/18-02-146.1

Tetzlaff, S., Allender, M., Ravesi, M., Smith, J., Kingsbury, B. (2015). First report of snake fungal disease from Michigan, USA involving Massasaugas, *Sistrurus catenatus* (Rafinesque 1818). *Herpetology Notes* 8: 31-33.

US Fish and Wildlife Service. (2011). Endangered and threatened wildlife and plants; removal of the Lake Erie watersnake (*Nerodia sipedon insularum*) from the federal list of endangered and threatened wildlife. Federal Register, 76(158), 50680.

Vissiennon, T., Schuppel, K-F., Ullrich, E., Kuijpers, A.F.A. (1999). Case report. A disseminated infection due to *Chrysosporium queenslandicum* in a garter snake (*Thamnophis*). *Mycoses* 42, 107-110.

Wellehan, J., Divers, S. (2019). Chapter 31: Mycology. In S. Divers & S. Stahl (Eds.), *Mader's Reptile and Amphibian Medicine and Surgery* (3rd ed., pp.275-276). Saunders.

Walker, D. M., Leys, J. E., Grisnik, M., Grajal-Puche, A., Murray, C. M., & Allender, M. C. (2019). Variability in snake skin microbial assemblages across spatial scales and disease states. *The ISME Journal*, *13*(9), 2209–2222. doi:10.1038/s41396-019-0416-x

Zimmerman, L. M., Vogel, L. A., & Bowden, R. M. (2010). Understanding the vertebrate immune system: Insights from the reptilian perspective. *Journal of Experimental Biology*, *213*(5), 661–671. doi:10.1242/jeb.038315