EFFECTS OF SNAKE FUNGAL DISEASE ON EPIDERMAL MICROBIOME DYNAMICS

by

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ABSTRACT

Ecological disturbance is a key factor in structuring biotic assemblages. Microbial assemblages are subject to disturbance and react predictably to it. This study aimed to determine if host- and pathogen- associated processes act as perturbations to the microbiome. Snake Fungal Disease (SFD), caused by *Ophidiomyces ophiodiicola*, is an emerging infectious disease. I hypothesized that shedding and disease would alter the structure and assembly of the snake microbiome. I inoculated 22 Northern Watersnakes (*Nerodia sipedon*) with *O. ophiodiicola* to assess the effects of SFD on host microbial assemblages over 82 days. Swabs were taken of each snake/mesocosm weekly and during shedding events. Swabs were utilized for quantitative PCR of *O. ophiodiicola* (pathogen load) and high-throughput sequencing to characterize microbial assemblages. Infection with SFD generated chronic disturbance of the microbiome; resulting in changes to alpha and beta diversity. Shedding generated acute disturbance of the microbiome; resulting in changes to beta-diversity and assembly processes. This work demonstrates the utility of disturbance ecology in studies of host-pathogen-microbiome interactions.

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CHAPTER I: INTRODUCTION

Emerging infectious diseases (EIDs) have been identified by conservation biologists as a leading threat to global biodiversity in this century (Daszak et al., 2000; 2001; Pedersen et al., 2007; Smith et al., 2006). Historically, infectious diseases were considered to be insufficient to cause extinctions in wildlife populations due to a correlation between host density and transmission rate (Anderson and May 1979; Getz and Pickering 1983). However, recent work has shown that EIDs act synergistically with environmental stress factors, such as habitat loss and introduction of exotic species; further reducing imperiled wildlife populations (Aguirre and Tabor 2008; Bradley and Altizer 2007; Castro and Bolker 2005; Hing et al., 2016). EIDs caused by fungal pathogens are of particular concern due to an increased risk of extinction or extirpation of their host species (Fisher et al., 2012). Fungal pathogens that cause EIDs infect a diversity of taxonomic groups, ranging from crop plants to tropical marine invertebrates (Fisher et al., 2012).

Fungal EIDs such as *Pseudogymnoascus destructans* (the causative agent of white nose syndrome) and *Batrachochytrium dendrobatidis* (a causative agent of chytridiomycosis) have had significant negative effects on bat and amphibian populations (Skerratt et al., 2007; Warnecke et al., 2012). White nose syndrome was first documented in New York in 2006 (Blehert et al., 2009). As a result of this pathogen, some hibernacula in the Northeastern United States experienced a 75% bat population decline within a two-year period (Blehert et al., 2009). Despite this, some bat populations are enduring the effects of white nose syndrome through the development of resistance or tolerance to the pathogen (Frick et al., 2016; Langwig et al., 2017). Chytridiomycosis first appeared, approximately simultaneously, in the

Americas and Australia during the 1970's resulting in the sudden decline of amphibian populations (Berger et al., 2016). However, chytridiomycosis was not characterized as the causative agent of these declines until the late 1990's (Berger et al., 1998). Chytridiomycosis has continued to negatively impact both salamander and frog populations around the world and is currently implicated in the decline or extinction of more than 200 species of amphibians (Skerratt et al., 2007; Voyles et al., 2009; Wake and Vredenburg 2008). Fungal EIDs are not restricted to mammals and amphibians. For example, a recently described EID, snake fungal disease (SFD), has been documented in free-ranging snake populations in the Eastern United States and Europe (Franklinos et al., 2017; Lorch et al., 2016). Cases of SFD have also been documented in the Western United States and tentatively in South America; potentially indicating a continued expansion of the geographic range of SFD (Bustos et al., 2018; CDFW 2019).

In 2006, individuals of the last known population of Timber Rattlesnakes (*Crotalus horridus*) in New Hampshire became afflicted with a disease that caused severe and often lethal infections (Clark et al., 2011). Between 2006 and 2007, a ~50% decline was documented in this Timber Rattlesnake population (Clark et al., 2011). This event has been cited as the first documented outbreak of SFD in a population of wild snakes (Allender et al., 2015b); however, the lack of molecular or microscopic identification of the causative agent has made it impossible to conclusively identify this as an occurrence of SFD (Clark et al., 2011). Further research has demonstrated that the fungus *Ophidiomyces ophiodiicola* is the causative agent of SFD (Lorch et al., 2015; Paré and Sigler 2016). *Ophidiomyces ophiodiicola* is a member of the order Onygenales which contains many keratinophilic species of fungi and human pathogens of some clinical significance (Koufopanou et al., 2001;

Paré and Sigler 2016; Sharma and Rajak 2003). *Ophidiomyces ophiodiicola* was originally classified within the genus *Chrysosporium*; which contains other cutaneous pathogens of reptiles (Paré and Sigler 2016); however, phylogenetic analysis of sequence data from the nuclear rDNA ITS and 18S regions led to the description of a new genus to describe *O. ophiodiicola* (Sigler and Paré 2013). Clinical signs associated with SFD include swelling of facial tissues, skin lesions/ulcerations, necrosis of the epidermis, lethargy, and increased frequency of shedding (Allender et al., 2015b; Lorch et al., 2015). During experimental infection of Cottonmouths (*Agkistrodon piscivorous*) with *O. ophiodiicola*, two of five snakes became sufficiently ill to warrant euthanasia over a 90-day study period, suggesting the lethality of this fungus (Allender et al., 2015a). The effect of SFD on wild snake populations is poorly understood; however, evidence suggests there is the potential for this disease to have a significant impact on snake populations (Lorch et al., 2015; 2016; Sutherland et al., 2014; Tetzlaff et al., 2017). For example, infection with *O. ophiodiicola* in a population of free-ranging Pygmy Rattlesnakes (*Sistrurus miliarius*) was correlated with increased resting metabolic rate and evaporative water loss (Agugliaro et al., 2019). In another study of the same population of Pygmy Rattlesnakes, SFD was associated with lower levels of testosterone in males during spermatogenesis and breeding potentially resulting in depression of reproductive success (Lind et al., 2019). Currently, the mode of transmission for SFD is not well resolved due, in large part, to an incomplete understanding of the life history of *O. ophiodiicola*.

Several hypotheses have been proposed to explain transmission of SFD including direct contact between uninfected and infected individuals and/or inoculation from environmental reservoirs (Lorch et al., 2016). It has also been

suggested that *O. ophiodiicola* may persist as a saprotroph in soil due to its ability to utilize a wide variety of carbon and nitrogen sources, tolerate a wide range of environmental pH, and endure water stress (Allender et al., 2015b). In at least one study, the molecular presence of *O. ophiodiicola* has been detected on the landscape from soil samples lending support to this hypothesis (Walker et al., 2019). Fungal pathogens that can engage in both saprotrophic and pathogenic life histories represent an especially high-risk to host populations (Fisher et al., 2012; Merikanto et al., 2012). The rate at which saprotrophic pathogens infect new hosts is somewhat decoupled from host population size allowing for chronic depression or extinction of host populations (Fisher et al., 2012; Merikanto et al., 2012). Additionally, host susceptibility to SFD shows a pattern of indiscriminate infection; potentially indicating widespread susceptibility of snake species in the United States (Burbrink et al., 2017). This is noteworthy as pathogens with multiple host species may exhibit both increased severity of outbreaks and persistence (Dobson 2004). Recent work has shown incidences of tentative vertical transmission of SFD (Stengle et al., 2019). For example, eggs laid by a female Eastern Kingsnake (*Lampropeltis getula*) with SFD were incubated separately from their mother, yet the neonates developed lesions after hatching (Stengle et al., 2019). Given the potential of SFD to exert both lethal and sub-lethal effects on wild snake populations, there is a need to develop a thorough understanding of its effects on host health.

Squamate reptiles, such as snakes, shed the upper layers of their epidermis on a regular basis. Shedding typically occurs on a monthly basis in snakes, with younger animals shedding more frequently (Semlitsch 1979). Host-response to infection with SFD includes an increased incidence of shedding (Lorch et al., 2015). During shedding events, snakes can rid themselves of necrotic tissue and pathogen mycelium

present on the old epidermis; often with a surprisingly high degree of apparent effectiveness (Lorch et al., 2015; Stengle et al., 2019). However, if pathogen hyphae colonize the developing epidermis before a shedding event takes place, then shedding, as a mechanism to clear *O. ophiodiicola*, may not be effective (Lorch et al., 2015). Additionally, infection with SFD can sometimes result in complications during the shedding process referred to as dysecdysis; especially in proximity to clinical signs (Lorch et al., 2015). Consequently, the host may need to undergo a series of rapid shedding events to successfully eliminate the pathogen from the epidermis (Lorch et al., 2015). To develop a comprehensive understanding of the host response to SFD, it is necessary to more fully characterize both the function and effects of shedding.

Many field studies examining SFD utilize single samples of individuals taken at a single time point Guthrie et al., 2016; Walker et al., 2019; Allender et al., 2018; Chandler et al., 2019). Field studies that have examined animals over multiple time points report significant variability in clinical signs and fungal load (predicted by quantitative PCR; qPCR) within individuals (Lind et al., 2018; 2019; Tetzlaff et al., 2017). Understanding the mechanisms that underlie this variability is an unresolved but central issue in the SFD literature. Some factors that have been implicated in this phenomenon include brumation and reproduction (McCoy et al., 2017; McKenzie et al., 2019). Shedding likely also has an impact on pathogen load and severity of clinical signs contributing to some of the individual-level variation being observed. During shedding events, infected tissue is discarded by the host. Consequently, pathogen load, as measured by qPCR, is likely to fluctuate when snakes undergo shedding. This could confound attempts to utilize qPCR to quantify disease severity in wild individuals; particularly in animals that have not been sampled repeatedly. Shedding is also likely to have effects on microbial assemblages known to exist on the

snake (host) epidermis (Allender et al., 2018; Walker et al., 2019).

The tissues and organs of multicellular organisms provide a dynamic habitat for microorganisms, giving rise to distinct microbial assemblages known as microbiomes (Huttenhower et al., 2012; Koenig et al., 2011). The epidermis of vertebrates harbors a diverse assemblage of microbes whose composition can be altered by factors such as microtopography, host demographics, and environmental parameters (Grice and Segre 2011). In addition to these factors, microbial assemblages are subject to ecological processes such as disturbance (Allison and Martiny, 2008; Atlas et al., 1991; Lozupone et al., 2012). Theory in disturbance ecology allows investigators to make predictions regarding how environmental perturbations will affect biotic assemblages (Rykiel et al., 1985). Perturbations to microbial assemblages include, but are not limited to, antibiotic administration in gut microbiomes (Lozupone et al., 2012), wildfire in soil microbiomes (Ferrenberg et al., 2013), and bioturbation in marine sediment microbiomes (Findlay et al., 1990). Furthermore, disease state and host-mediated factors, such as obesity, pregnancy, and host behavior, are correlated with changes in the host microbiome (Cho and Blaser 2012; Ezenwa 2012; Kong et al., 2012; Koren et al., 2012; Turnbaugh et al., 2006; Wegner et al., 2013). Thus, it is plausible that both SFD (disease state) and shedding events (host-mediated factor) represent disturbance events to the epidermal microbiome of snakes. Consequently, disturbance ecology can be utilized to generate hypotheses concerning the impacts of these events on the structure and assembly of the host microbiome.

The total number of species present within an assemblage can be defined as the richness of that assemblage, making it one of the more intuitive diversity indices (Hill et al., 2003). Alpha diversity metrics quantify the evenness and richness of a

particular assemblage with different alpha diversity metrics (Shannon, Inverse Simpson, etc.) placing more weight on one component over the other (Hill et al., 2003). Beta diversity metrics compare all of the different assemblages in a logical group (Tuomisto 2010). A group that contains more dissimilar assemblages relative to each other will have a higher value for beta diversity than a group that contains more similar assemblages relative to one another (Tuomisto 2010). Consequently, these types of metrics have been referred to as "turnover diversity" and include Sorenson and Simpson indices (Tuomisto 2010). Along with these analytical methods for describing assemblage structure, conceptual models have been proposed regarding the assembly of biotic systems. The assembly of biotic systems can be described by the processes of speciation, dispersal, drift, and selection (Nemergut et al., 2013; Vellend 2010). Within this framework, new species are added to assemblages through the processes of speciation and dispersal (Vellend 2010). Drift and selection alter the relative abundance of species already present within an assemblage (Vellend 2010). Previous work has demonstrated that the epidermal microbiome of snakes sampled in the Eastern United States is distinct from environmental microbial assemblages (Leys 2017). Furthermore, host species, host habitat, and the presence of *O. ophiodiicola* are predictive of the snake microbiome across spatial scales (Walker et al., 2019). This suggests that the microbial assemblage present within the snake epidermal microbiome is not simply a product of random dispersal of microbes from the environment onto the epidermis. Rather, microbial assemblages present on the snake epidermis are likely formed via the same ecological processes that shape macroscopic assemblages.

SFD correlates with changes in the epidermal microbiome (Allender et al., 2018; Walker et al., 2019), and changes in biotic systems are often mediated by

disturbance. Therefore, I expect SFD to act as a chronic disturbance to the epidermal microbiome of snakes. Chronic microbiome disturbance may persist for several years given that wild snakes can tolerate yearly SFD infection (Lind et al., 2018; Tetzlaff et al., 2017). Over the course of infection, this disease may alter the physical and chemical environment of the epidermis. *Ophidiomyces ophiodiicola* has been shown to produce enzymes, such as keratinase and gelatinase, that are capable of digesting some of the molecular constituents of epidermal tissues (Allender et al., 2015b). The extent of necrosis, dermatitis, and hyphal proliferation typically increases over the course of infection, thereby increasing the potential for pathogen-mediated changes to the epidermal environment (Lorch et al., 2015). Fungal pathogens of plants act in a similar manner, using hyphal secretion of proteins and toxins to induce dieback and necrosis within host tissues (Kolattukudy 1985; Ramírez-Suero et al., 2014). Therefore, it is likely that pathogen-mediated changes to the epidermal environment will become more prominent over the course of infection. Thus, as infection progresses there will be cumulative changes in the availability of physical and chemical resources for epidermis colonizing microbes. Consequently, I expect that if SFD effects the microbiome in the manner anticipated, it will alter assembly to enhance the effects of selection (e.g. environmental filtering).

Selection can be described as a deterministic variation in fitness between individuals within an assemblage (Vellend 2010). For selection to take place on the microbiome, several assumptions would need to be met including: 1) variability in the fitness of microbial taxa, and 2) heritability of traits which affect fitness (Vellend 2010). In microbial assemblages, selection acts more readily on functional groups rather than taxonomic groups, due in part to horizontal gene transfer among microbes (Nemergut et al., 2013). However, research shows that evolutionary relatedness and

ecological function are often still directly related to one another (Fuhrman 2009). Therefore, by studying variation among taxa within a microbial assemblage, it is possible to make inferences about how selection may be acting on a system (Fernandez et al., 2000; Ju et al., 2014). Because selection is a deterministic process, I expect disease state to reflect predictable patterns of microbial assembly. More specifically, I hypothesize that microbiome structure of SFD infected animals will be more similar to one another, relative to uninfected animals. The microbiome of snakes infected with SFD will ultimately be influenced by both natural (e.g. shedding) and pathogen-mediated processes. The relative strength of the deterministic processes resulting from SFD compared to stochastic assembly processes will determine if disease status is sufficient to generate distinct assemblages on infected animals.

Unlike disease, which likely represents a chronic disturbance to the microbiome, shedding typically occurs over a period of several hours. Consequently, it may be more appropriate to conceptualize shedding as an acute disturbance. During a shedding event, a significant amount of microbial habitat (epidermis), and presumably microbes as well, are lost from the system in a small temporal window (approximately 1-3 hours, determined herein). If this occurs, microbial assemblages that are reduced in abundance will be particularly vulnerable to the effects of drift. Ecological drift is the non-deterministic change in the relative abundances of species within an assemblage, resulting from the stochastic components of reproduction (Vellend 2010). When population sizes are small, the impact of selection on assemblage is reduced, but the impact of drift is enhanced (Stegen et al., 2012; Vellend 2010; Zhou et al., 2013). Therefore, if microbial abundances are significantly reduced during shedding, then skin taxa may be more subject to the effects of drift. I would expect skin assemblages to more closely resemble a null expectation of beta

diversity, because changes in assemblage structure generated by drift are stochastic in nature (Vellend 2010). Consequently, the structure of biotic assemblages affected by drift alone should not be more or less similar then would be expected by random chance (Chase and Myers 2011). Alternatively, assemblages may become more similar or dissimilar than would be expected from stochastic processes alone due to the effects of selection and dispersal (Dini-Andreote et al., 2015).

Another assembly process likely to structure the epidermal snake microbiome is dispersal, which is defined as the movement of organisms through space (Vellend 2010). In some microbial assemblages (e.g. pelagic marine), increased dispersal of taxa can increase similarity of assemblage structure (Wilkins et al., 2013). Modulation of dispersal in other bacterial assemblages (e.g. plant litter) has been correlated with shifts in assemblage richness and composition (Albright and Martiny, 2018). Consequently, to predict its effects, dispersal must be considered from the ecological context in which it occurs. I predict the effects of dispersal to be prominent within the snake microbiome during shedding. If microbial taxa are lost from the epidermal environment as a result of shedding, dispersal may determine the ability of a taxon to recolonize freshly exposed snake skin. Thus, continuity of the snake microbiome may be determined by the dispersal ability of taxa found within this system. Microbial taxa are normally considered to be robust dispersers given their small size, rapid reproduction, and high cellular abundance in ecosystems (Nemergut et al., 2013). An assumption of neutral dispersal ability is often made in regard to microbes wherein dispersal limitation equally affects all taxa (Nemergut et al., 2013). This assumption exists because it is difficult to measure the impact of dispersal limitation on microbial assemblages (Nemergut et al., 2013). Thus, the distribution of microbial taxa is often used as a proxy for dispersal, which does not account for the

exclusion of microbes from an environment via environmental filtering (Nemergut et al., 2013). If the assumption of neutral dispersal ability among microbes is accepted, both drift and dispersal would act as stochastic processes within the snake microbiome. Consequently, shedding in snakes would primarily exert stochastic forces of assembly resulting in non-deterministic changes to the composition of the microbiome. I expect this to be the case regardless of infection status.

The overall objective of this project is to demonstrate how SFD and shedding events may act as disturbances to the epidermal microbiome of snakes thereby altering structure and assembly of this system. My specific objectives are to 1) determine how the process of shedding effects the structure and assembly of the microbiome; 2) determine how SFD effects the structure and assembly of the microbiome and; 3) determine the degree of microbial depletion in the epidermal microbiome that may occur as a result of shedding. Disease state is expected to generate consistent selective pressures across the microbiomes of infected snakes as the epidermal environment is enzymatically modified by the pathogen. Consequently, I expect to observe consistent changes to the structure of the microbiomes of diseased snakes relative to healthy snakes resulting from pathogen-mediated changes to the epidermal environment. The effect of deterministic processes like selection are expected to supersede the effects of non-deterministic processes like drift in infected snakes. Thus, I hypothesize that the assemblage structure of control snakes will show less deterministic variation and subsequently more closely adhere to a null model of beta-diversity. Alternatively, I hypothesize that infected snakes will show more deterministic variation in the structure of their microbiomes and subsequently less closely adhere to a null model of beta-diversity. I predict that richness of the microbiome will be reduced in both experimentally infected and control snakes on

newly exposed epidermis immediately after shedding events. I predict that detectable pathogen load will be reduced in experimentally inoculated snakes as a result of shedding events. Results of this experiment will help to determine if changes in the microbiome of snakes take place in a continuous fashion (shedding has little effect on the epidermal microbiome) or a discontinuous fashion (shedding has a significant effect on the epidermal microbiome). This will provide a long-term temporal framework for interpreting snake microbiome dynamics that incorporates assembly processes, disturbance ecology, and host behavior. Additionally, results will elucidate how the epidermal microbiome of snakes is altered by SFD over a clinically relevant timeframe.

CHAPTER II: METHODS

Study Species

The Common Watersnake (*Nerodia sipedon*) is a nonvenomous semi-aquatic snake found throughout Eastern and Central North America (Mebert 2008). A study of *N. sipedon* in Eastern Ontario found that individuals typically grew to a total length of 70–90 cm and reached a weight of 100-300 g (Weatherhead et al., 1995). Within their range, this species is commonly found in and around bodies of water such as streams, ponds, bogs, swamps, and marshes (Powell et al., 2016). Frogs and fishes were found to make up the majority of the diet in dissections of both large (> 50cm) and small (< 50cm) preserved specimens of *N. sipedon* demonstrating the importance of this species as an aquatic predator (Himes 2003). *Nerodia sipedon* is characterized as a species of least conservation concern due to its high population densities, wide distribution, and tolerance of habitat modification (IUCN 2007).

Snake/Soil Collection

A total of 26 *N. sipedon* were collected in from populations in Tennessee during the Spring of 2019 with TWRA approval (Scientific Collection Permit #1907). Snakes were collected in Rutherford, Cheatham, and Putnam counties using hand capture methods (Table 1). All handling of snakes was completed using nitrile gloves to prevent the transmission of SFD or other pathogens between animals during the collection process. After capture, snakes were immediately examined for clinical signs of SFD, such as gross skin lesions or mycosis. Only snakes free of clinical signs were considered for use as experimental subjects to control for disease state. Collected snakes free of clinical signs were held for a maximum of 24 hours to allow for quantitative PCR (qPCR) detection of *O. ophiodiicola* (Bohuski et al., 2015).

Snakes positive for *O. ophiodiicola* were returned to their capture locations. Four of the 26 snakes collected were omitted from the study and released due to the detection of *O. ophiodiicola* . Snakes negative for *O. ophiodiicola* were utilized as experimental subjects. A two-liter bag of soil was collected approximately three meters in a random direction closest to the river edge from each snake capture location. Soil samples were also tested for the presence of *O. ophiodiicola* via qPCR assay. Quantitative PCR (qPCR) results indicated that all soil samples collected during initial field work were negative for *O. ophiodiicola*; therefore, all soils from point capture locations were used in construction of mesocosms. Soil samples were stored at 4°C from the day of collection until the start of live animal trails. Soil samples were utilized in experimental mesocosms to mimic an environmental reservoir of microbes.

Mesocosm Design

Snakes were maintained individually in 66.24 L plastic storage totes (66 × 34 × 41 cm) with ventilation holes. Each enclosure was supplied with a hide box, climbing branch, a water dish, and soil/aspen substrate mixture. Equal parts, by weight, of each soil sample collected on the landscape were combined with aspen snake bedding to form a 2:1 soil to aspen ratio by weight. Aspen snake bedding and climbing branches were autoclaved for 2 hours prior to use in the experiment to limit the addition of unwanted microbial taxa. The mixture was then used to form a substrate layer ~6 cm deep in all mesocosms. When soiled, substrate was spot cleaned throughout the clinical trial and replaced completely whenever snakes spilled water onto the substrate. Water dishes were cleaned weekly or whenever the water was fouled. Enclosures were located in a 23°C room with a 12-hour light/dark cycle. Depending on animal size, snakes were offered as many Guppies (*Poecilia reticulata;*

small snakes; > 35cm SVL) or Platies (*Xiphophorus maculatus;* large snakes; < 35cm SVL) as they would consume weekly.

Live Animal Trials

Animal trials began on 31 May 2019 and concluded on 21 August 2019 (IACUC approval 19-3012). Eleven snakes were randomly assigned to the treatment group and eleven to the control group. The snakes in the treatment group were inoculated with *O. ophiodiicola* using the following procedure. A culture of *O. ophiodiicola* was grown on Sabouraud dextrose agar (SDA) for 15 days and sectioned into 0.5 cm^2 portions then placed, mycelium side up, onto a waterproof bandage. Similar to Lorch et al., (2015), I used #150 sandpaper to abrade, using five strokes, the dorsal, ventral, and neck surface of the skin of each snake. Bandages with *O. ophiodiicola* and SDA were placed on each abrasion site to inoculate the skin for 72 hours before being removed. Animals in the control group received the same treatment although sterile SDA cubes were applied to the bandages. Each snake was observed on a daily basis to perform general health checks and monitor their shedding cycles. Euthanasia was accomplished at the end of the clinical trial by injection with sodium pentobarbital.

Every seven days, I collected samples of the epidermal microbiome for all 22 individuals; this dataset is referred to as the weekly sampling dataset throughout the manuscript. Aseptic technique was used when work was carried out in and around the enclosures to ensure that *O. ophiodiicola* and other microbes were not transferred between enclosures. The swabbing protocol utilized to collect microbial samples involved spraying a rayon-tipped sterile applicator (Puritan, VWR cat #10808-146) with 2 hour autoclaved Millipore water for five seconds. This applicator was then

rotated over a 15 cm portion of the snake's midbody 15 times (similar to Walker et al., 2019). For environmental samples, the applicator was rotated over a 15 cm portion of the substrate surface 15 times. Additionally, snout-vent length and weight was recorded weekly for each animal.

During the experiment, snakes were placed under near constant observation (~900 hours in total) to monitor for shedding events by study animals. Six shedding events were successfully sampled. This level of observation was maintained throughout the summer for 83 total days, until 21 August 2019 when the experiment ended, and surviving animals were euthanized via sodium pentobarbital. Sampling of the microbiome was performed during shedding events in a timeseries fashion. During each sampling event, swabs of the cutaneous and environmental microbiome were performed. Microbial samples were collected during the shedding process and then in a timeseries (0, 1, 2, 4, 8, 12, and 24 h after shedding); this dataset is referred to as the short-term shedding timeseries throughout the manuscript. To sample the skin surface not yet exposed to microbial colonization from the environment (mid-shedding at time point 0), the old epidermis was manually peeled back 15 cm, and the swab was rotated across the freshly exposed epidermis using 15 strokes. If the old epidermis could not be pulled back 15 cm, the swab was moved over the newly exposed epidermis an appropriate number of times to standardize the sampled surface area (i.e. 5 cm ~ 45 strokes). All swab samples were stored at -20°C until DNA extraction. Swabs collected during weekly and shed sampling were used to characterize assemblage structure and quantify fungal load of *O. ophiodiicola*. For modeling purposes, swabs associated with shedding events were divided into two distinct sampling categories: 1) a short-term shedding timeseries which included only the samples collected in the hourly series (0, 1, 2, 4, 8, 12, and 24 hrs) following

shedding, and 2) a long-term shedding timeseries which included samples collected during the weekly sampling prior to shed, during shedding, and in the weekly sampling event post shed.

Host Body Condition

Morphometric data (SVL and weight) collected throughout the study was used to calculate body condition of snakes. Body condition indices are commonly used a proxy for animal health in ecological literature (Jakob et al., 1996). The scaled mass index (SMI), a widely utilized metric for calculating body condition, was applied in this study as a proxy for host health (Peig and Green 2009). Research has validated that this metric is effective at estimating snake body condition (Falk et al., 2017). Calculated SMI values were normalized using a Tukey's ladder of powers transformation. Normalized SMI values were modeled using a linear mixed-effect model in the R package *lme4* (Bates et al., 2014). Probabilistic model selection was preformed using Akaike Information Criterion (AIC) values (Bozdogan 1987). Sampling week and animal identity were included as crossed random effects in the most likely model. Animal identity is a factor which specifically identifies each subject animal to the model such that between-subject differences can be explicitly accounted for. This factor is used for modeling purposes throughout the manuscript.

Quantifying Pathogen Load

DNA was extracted from skin and soil swab samples using the Qiagen DNeasy PowerSoil kit per the manufacturer's protocol. On each 96 well plate, a single DNA negative control blank was extracted to filter out contamination during qPCR and bioinformatics analyses. Pathogen load was measured using qPCR of the ITS region of the rRNA gene of *O. ophiodiicola* (Bohuski et al., 2015). Reactions and

criteria for detection of positive samples followed the methods described by Walker et al., (2019). Any control animal that had a positive qPCR reaction throughout the experiment was removed from all analyses. To determine pathogen load within each sample, a serial dilution of $1 – 1 \times 10^{10}$ copies of a synthetic DNA qPCR target sequence (gBlock, IDT), was created and used to generate a standard curve. The log copy number of *O. ophiodiicola* per qPCR reaction was calculated using the formula $y = -0.2893x + 10.783$, which was generated from the slope of the standard curve. The value 'x' was the average C_t for each unknown sample run in triplicate. The copy number values calculated for all qPCR positive samples were log transformed and then rounded up to the nearest integer value for modeling with a Poisson distribution. Copy number values were used to predict if pathogen load in inoculated animals was predictive of host health. Modeling was accomplished using a generalized linear mixed-effects model with a zero-inflated Poisson distribution in the software package *glmmTMB* (Brooks et al., 2017). Model selection was preformed using Akaike Information Criterion values (Bozdogan 1987). Sampling week and animal identity, treated as crossed random effects, were included in the most likely model. Time until death and animal length were included as fixed effects in the most likely model. Death was defined as the date at which an individual experienced mortality over the clinical trial or was euthanized because the experiment concluded.

Pathogen load was also examined using qPCR over the course of shedding events in inoculated animals. For this modeling application, the long-term shedding timeseries was utilized because changes in disease dynamics are likely to occur over longer temporal scales. Swab collection times were converted into units of days post-shed to allow for linear modeling. This provided a continuous vector of time along which *O. ophiodiicola* copy number values could be modeled to determine the effect

of shedding on pathogen load. This was accomplished using a generalized linear-mixed model with a Poisson distribution in the R package *lme4* (Bates et al., 2014). Animal identity was specified as a random effect in this model.

Amplicon Sequencing and Bioinformatics

Using the isolated DNA from skin swabs, a region of the 16S rRNA marker was amplified using primers 806R and 515F (Caporaso et al., 2011). Amplicons were dual indexed following the protocol described in Fadrosh et al. (2014). Indexed amplicons were selected based on fragment size to remove adapter dimers and retain PCR product using MagBio HighPrep magnetic beads. The concentration of each library was quantified using a Quantus fluorometer, normalized, and pooled before sequencing on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA) using eight independent 2 × 250 bp paired end runs to generate 95 million sequences.

The software platform Mother v1.43.0 was used to conduct bioinformatic analyses of amplicon sequencing data (Schloss et al., 2009) according to the MiSeq SOP (Kozich et al., 2013) with several modifications. After forming contigs, *screen.seqs* was used to trim sequences to primer locations. Sequences with a minimum of 248 bp and maximum length of 256 bp were then selected for downstream analysis . Sequences with ambiguous base calls and homopolymers greater than eight were removed from the data set. Remaining sequences were aligned to the SILVA v132 reference alignment (Quast et al., 2013; Yilmaz et al., 2014). The data were denoised using the *pre.cluster* command to merge sequences with two or fewer nucleotide differences. The *chimera.vsearch* command was used to remove chimeric sequences using the parameter ‘template=self’. Sequences identified as chloroplast, mitochondria, unknown, Archaea or Eukarya were removed from the

dataset. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using the *cluster.split* command. OTUs identified in negative control sequencing blanks (1602 total OTUs) were removed from the final data set. Rare OTUs (< 5) were removed using the *remove.rare* command and 'bygroup=T' option to remove any OTU that had fewer than the threshold sequences (< 5) on a per sample basis. Samples were normalized by subsampling at 1102 sequence reads (Weiss et al., 2017) to generate the final dataset that was imported into R for statistical analysis.

CHAPTER III: STASTICAL METHODS

Chronic Disturbance – Microbial Assemblage Alpha Diversity

The weekly time series samples (n = 144) were used to determine the effect of *O. ophiodiicola* as a chronic disturbance to skin assemblages. Species richness was calculated using the "specnumber" function in the package *vegan*. Species richness was fit to a normal distribution using a Tukey's Ladder of Powers transformation. Species richness was examined across weekly samples of the microbiome to understand the chronic effects of disease on microbial richness using a linear mixed-effects model in the package *lme4*. Model selection was preformed using Akaike Information Criterion (AIC) values (Bozdogan 1987). Sampling week and animal identity were treated as crossed random effects in the AIC selected model.

Alpha diversity was calculated using the "diversity" function with the index "shannon" in the package *vegan* (Dixon 2003). Shannon-Weaver values were fit to a normal distribution using a Tukey's ladder of powers transformation and modeled using a linear mixed-effect model in the package *lme4* (Bates et al., 2014). A quadratic function, using the "poly" option in the package *stats,* was used to determine the relationship between time and alpha diversity as it appeared to be non-linear. Sampling week and animal identity were treated as crossed random effects in the model.

Chronic Disturbance – Turnover and Nestedness

The structure of microbial assemblages was examined using the *betapart* package which can scale an OTU abundance matrix into separate pairwise dissimilarity matrices representing total beta diversity, turnover and nestedness (Baselga and Orme 2012). To understand *O. ophiodiicola* as a chronic disturbance to assemblage

structure, weekly snake microbiome samples were scaled to presence/absence data using the function “decostand” in the package *vegan* (Dixon 2003). This matrix was converted into a betapart object using the “betapart.core” function and then partitioned into separate components of beta diversity using the Sorenson Dissimilarity index (“beta.pair” function). This generated a separate distance matrix for total beta diversity, turnover beta diversity, and nested beta diversity. The function “mixed.mdmr” from the package *MDMR* (McArtor 2016) was used to perform mixed multivariate distance matrix regression to determine the significance of fixed effects regressed against the matrix while accounting for random effects. Field capture location and animal identity were utilized as random effects with animal identity being nested within capture location. Parameters selected as fixed effects were experimental group, time, the interaction between experimental group and time, body condition, and the interaction between experimental group and body condition. The Bonferroni correction was utilized in all tests of mixed multivariate distance matrix regression (both here and below) to ensure that multiple comparisons were conservatively corrected for (Armstrong 2014). The initial alpha value used for all Bonferroni correction values was set at 0.05. Adjusted alpha values were reported for all mixed multivariate distance matrix regression models in the results section. Distance-based redundancy analysis, using the function “capscale” in the package *vegan* (Dixon 2003), was utilized to create a constrained ordination of total and turnover beta-diversity using significant response variables identified with mixed multivariate distance matrix regression modeling. The significance of individual constraints was evaluated using a type III test (function “anova”) to account for correlation between independent variables.

Weekly snake microbiome samples from inoculated animals (n = 84) were also

examined separately to determine if components of clinical outcome, such as host body condition, effect beta diversity of the host microbiome. A distance matrix for turnover beta diversity was analyzed with multivariate distance matrix regression and visualized as a constrained ordination using distance-based redundancy analysis as previously described. More specifically, fixed effects in this model included log-transformed copy number, time, body condition, pathogen presence, time until death, clinical survival, the interaction between time and pathogen presence, and the interaction between clinical survival and time until death. Field capture location and animal identity were utilized as random effects with animal identity being nested within capture location.

Acute Disturbance – Microbial Assemblage Alpha Diversity

Shed timeseries samples were utilized to determine the effect of *O. ophiodiicola* as an acute disturbance to skin assemblages. Using the long-term timeseries (n = 41), time was converted into units of "days-post-shed" to model changes in alpha diversity in a linear fashion against a continuous variable. Consequently, samples taken at the time of shedding would be assigned a temporal value of 0, samples taken 12 hours after shedding would be a assigned a value of 0.5, and samples taken 24 hours after shedding would be assigned a value of 1. The temporal value of post- and pre- shed samples was assigned to each sample based on when each shedding event occurred relative to regular weekly sampling. Pre-shed samples always had a value of -1 (or less), while post-shed samples always had a value of 1 (or greater). Species richness was calculated as previously described and normalized using a Tukey's ladder of powers transformation. A linear mixed-effect model was utilized to determine the effect of the long-term shedding process on

assemblage richness using the package *lme4* (Bates et al., 2014). Model selection was performed using Akaike Information Criterion values (Bozdogan 1987). The most likely model included animal identity as a random effect and the interaction between experimental treatment and days-post-shed as fixed effects.

The short-term (n = 33) timeseries was also utilized to determine the effect of the shedding process on skin assemblages with time being treated in the same fashion as described above. The most likely model included animal identity as a random effect and the interaction between experimental treatment and days-post-shed as fixed effects. Shannon-Weaver values were calculated as previously described and normalized using a Tukey's ladder of powers transformation. A linear mixed-effect model in the package *lme4* was utilized to determine if shedding, as a short-term process, was predictive of alpha diversity in the host microbiome. Time was modeled in the same fashion as described above. The most likely model included animal identity as a random effect and the interaction between experimental treatment and days-post-shed as fixed effects.

Acute Disturbance –Turnover and Nestedness

The short-term shedding timeseries was used to determine if shedding causes an acute disturbance to the structure of the skin microbiome. Sorenson distance matrices were generated, as previously described, using the packages *vegan* and *betapart* (Baselga and Orme 2012; Dixon 2003). Mixed multivariate distance matrix regression (McArtor 2016) was utilized to determine if inoculation status, experimental time, clinical survival, and time post-shed, set as fixed effects, were descriptive of variation in total beta diversity, turnover, or nested diversity. For these three models, capture location and animal identity were included as nested random effects with animal

identity being nested inside of capture location.

The long-term shedding series was also investigate impacts of the shedding process on the structure of the skin microbiome over a larger temporal scale. Specifically, samples were categorized as 1) “Pre-Shed”, 2) “Shed”, 3) or “Post-Shed”. This allowed communities to be compared based on their temporal position within the shedding process. As such, structural changes in microbial assemblages generated by shedding events could be detected. Distance matrices for beta diversity and turnover diversity were generated and mixed multivariate distance matrix regression was utilized as previously described to assess variation in community structure through the shedding process. Mixed multivariate distance matrix regression (McArtor 2016) was utilized to determine if inoculation status, shed state, or the interaction between inoculation status and shed state were descriptive of variation in total beta diversity or turnover diversity. For both models, capture location and animal identity were set as nested random effects with animal identity nested within capture location.

Null Deviation Models of Beta Diversity

Assembly of microbial assemblages can be studied through the use of null models of beta-diversity to test the likelihood that assemblages arise through the random sorting of taxa from a global taxon pool (Chase et al., 2011). The expected probability of a taxon being observed in any given site is a function of the global occurrence of that taxon across all sites (Chase et al., 2011). A null deviation metric broadly predicts that very common taxa would be found at both sites within a pair of assemblages and very rare taxa would not be found at either (Chase et al., 2011). If a rare taxon is found within a pair of assemblages, this would be an example of a deviation from the

neutral (i.e. probabilistic) model. Understanding these patterns allows for the prediction of assembly processes structuring assemblages (deterministic or stochastic) rather than simply comparing assemblage structure itself. The Raup-Crick metric is an example of this type of null beta-diversity metric. It was originally created to compare fauna among fossilized assemblages (Raup and Crick 1979). It has since been adopted and modified for use in microbial ecology (Chase et al., 2011). The modified Raup-Crick generates a dissimilarity matrix of sites, where pairwise comparisons between sites that exhibit no deviation from the null expectation, would be assigned a value of zero (Chase et al., 2011). Pairwise comparisons deviating from the null expectation are assigned a value between one and negative one, where a larger deviation from zero indicates greater variance from the null expectation (Chase et al., 2011). A positive value indicates that the two sites being compared are more dissimilar than would be expected due to random processes (Chase et al., 2011). A negative value indicates that the two sites being compared are more similar than would be expected due to random processes (Chase et al., 2011).

The computational requirements associated with the Raup-Crick metric greatly increase with an increase in the number of species added to an analysis. Consequently, samples selected for analysis were reduced by subsampling the most abundant OTUs in the 90th percentile of the snake microbiome during weekly and shedding samples. Samples from weekly (chronic disturbance) and shedding (acute disturbance using short-term time series) sampling events were analyzed separately. Both data sets were separately transformed into a presence/absence matrix and then examined using the modified Raup-Crick metric to generate a pairwise dissimilarity matrix. For weekly samples, mixed multivariate distance matrix regression was utilized to determine if inoculation status, time, and the interaction between

inoculation status and time, set as fixed effects, were descriptive of community assembly while controlling for animal identity as a random effect. A distance-based redundancy analysis was utilized as previously described to generate a constrained ordination for the Raup-Crick metric and its predictor variables. For short-term shedding samples, mixed multivariate distance matrix regression was utilized to determine if hour-post-shed, inoculation status, and the interaction between hour-post-shed and inoculation status, set as fixed effects, were descriptive of community assembly while controlling for animal identity as a random effect. Distance-based redundancy analysis was also utilized to generate constrained ordination of the results of this analysis.

CHAPTER IV: RESULTS

Host Health Implications

Linear mixed-effects modeling indicated that the control population had significantly higher host body condition relative to the inoculated population (LMM, t-value = 4.549, $p < 0.0005$, marginal $R^2 = 29.1\%$). A generalized linear mixed-effects model with a zero-inflated Poisson distribution indicated that higher pathogen loads, as measured by qPCR, were associated with a shorter time until death (GLMM, z-value = -2.581, $p < 0.01$, pseudo-$R^2 = 13.6\%$). A generalized linear mixed-effects model fitted with a Poisson distribution indicated that the process of shedding in snakes is correlated with a significant decrease in pathogen load (GLMM, z-value = -3.271, $p < 0.05$, marginal $R^2 = 12.4\%$).

Chronic Disturbance – Microbial Assemblage Alpha Diversity

Linear mixed-effects modeling indicated that community richness of control snakes was significantly lower than the assemblages of inoculated animals (LMM, t-value = -2.541, $p < 0.05$, marginal $R^2 = 6.5\%$). Additionally, a significant relationship was found between richness and the interaction between inoculation status and body condition (LMM, t-value = 3.105, $p < 0.005$, marginal $R^2 = 6.5\%$, Fig. 1). Control snakes had a positive relationship with host body condition, as predicted by scaled mass index, and community richness; whereas, inoculated snakes had a negative relationship with these variables. Linear mixed-effects modeling indicated a significant negative quadratic relationship between Shannon-Weaver diversity and time (LMM, t-value = -3.667, $p < 0.005$, marginal $R^2 = 12.4\%$). The distribution of Shannon-Weaver diversity values initially rose, reached an apex, and then declined as time progressed.

Chronic Disturbance –Turnover and Nestedness

Mixed multivariate distance matrix regression modeling indicated a significant relationship between total beta diversity and the interaction between body condition and inoculation status (MMDMR, t-stat = 4.12, df = 2, alpha = 0.01, $p < 0.01$, Fig. 2). A significant relationship was also found between assemblage turnover and the interaction between body condition and inoculation status (MMDMR, t-stat = 4.60, df = 2, alpha = 0.01, $p < 0.005$, Fig. 2) suggesting the disruption of typical feedback mechanisms between host health and the microbiome during infection with SFD. Mixed multivariate distance matrix regression modeling indicated a significant relationship between microbial turnover in inoculated snakes and time (MMDMR, t-stat = 4.26, $p < 0.0005$, alpha = 0.006), as well as, clinical survival (MMDMR, t-stat = 3.96, $p < 0.0005$, alpha = 0.006). These results suggest that the turnover of inoculated snake microbiomes may be correlated with the clinical outcome of the host.

Acute Disturbance – Microbial Richness and Alpha Diversity

Across the long-term shedding timeseries, linear mixed-effects modeling indicated that time had no significant effect on richness of the snake microbiome regardless of inoculation status (LMM, t-value = -0.629, $p > 0.05$, marginal R^2 = 10.7%). Similarly, no significant relationship was found between time and richness (LMM, t-value = -0.104, $p > 0.05$, marginal R^2 = 10.1%) for the short-term timeseries. Across the short-term shedding timeseries, linear mixed-effects modeling indicated that time had no significant effect on Shannon-Weaver diversity of snake microbiomes regardless of inoculation status (LMM, t-value = -0.326, $p > 0.05$, marginal R^2 = 13.8%).

Acute Disturbance –Turnover and Nestedness

During the short-term shedding timeseries, inoculation status had a significant effect on total beta diversity (MMDMR, t-stat = 10.9, $p < 0.0001$, alpha = 0.0125), turnover (MMDMR, t-stat = 33.43, $p < 0.0001$, alpha = 0.0125), and nestedness (MMDMR, t-stat = 5.23, $p < 0.005$, alpha = 0.0125). Over the course of the long-term shedding series, the interaction between inoculation status and shed state (pre-shed, shedding-process, post-shed) had a significant effect on total beta diversity (MMDMR, t-stat = 8.10, $p < 0.001$, alpha = 0.0167). Constrained ordination and distance-based redundancy analysis revealed a significant difference between beta-diversity of microbial assemblages from pre-shed and shed assemblages in both inoculated and control animals. This result suggests that the snake skin microbiome structure is altered during shedding events and the effects of shedding is contingent upon disease state. Turnover diversity was significantly altered by shed state (MMDMR, t-stat = 8.40, $p < 0.0001$, alpha = 0.0167, Fig. 3) during the long-term shed series. Constrained ordination revealed a significant difference in the degree of turnover diversity prior to shedding events and during events in a fashion that was not dependent on disease state (Fig. 3).

Null Deviation Models of Beta Diversity

In the weekly sampling dataset, inoculation status (MMDMR, t-stat = 2.78, $p < 0.015$, alpha = 0.0167) and the interaction between inoculation status and experimental week (MMDMR, t-stat = 2.85, $p \leq .01$, alpha = 0.0167, Fig. 4) had a significant relationship with the Raup-Crick metric (Fig. 4). In ordinations of typical beta diversity indices, the size of an ellipse is informative as to the relative similarity of assemblage structure among a group. Groups with more similar structure across all

communities will be represented by smaller ellipses and groups with less similar structure overall are plotted as larger ellipses. With regard to the Raup-Crick metric, ellipse size is informative to the nature of assembly rather than assemblage structure (Chase 2010). Specifically, groups that are more deviant from the null expectation will be plotted using smaller ellipses and groups less deviant from the null expectation will be plotted using larger ellipses. The ellipse sizes of control and inoculated snakes were approximately equal in a constrained ordination generated using distance-based redundancy analysis and the Raup-Crick metric (Fig. 4). This indicates that during the 'weekly sampling' dataset, the microbial assemblages found on inoculated snakes were not more or less deviant from the null expectation, relative to control snakes.

In the acute disturbance dataset, a significant relationship was found between the Raup-Crick metric and the interaction between inoculation status and time post-shed (MMDMR, t-stat = 4.15, $p < 0.005$, alpha = 0.0167, Fig. 5). This analysis revealed that inoculated snakes had less multivariate dispersion relative to those of control snakes. This indicates that during shedding, microbial assemblages found on inoculated snakes were more deviant from the null expectation, or had a higher prevalence of deterministic assembly processes, relative to control snakes.

CHAPTER V: DISCUSSION

Disturbance ecology allows investigators to predict and interpret the effects of perturbations, such as disease, on biotic assemblages (Rykiel 1985). In this investigation, SFD and shedding were investigated as potential chronic and acute disturbance events, respectively, to the host microbiome. By inoculating snakes with *O. ophiodiicola* in a controlled and pseudo-naturalistic setting, the effects of SFD and shedding could be examined to determine if these processes generate disturbance in the microbiome. I found several notable effects of disease on host health. These results contribute to a growing body of literature which suggests that infection with SFD has both lethal and sub-lethal consequences (Agugliaro et al., 2019; Lind et al., 2019; Lorch et al., 2016; Tetzlaff et al., 2017). Significant relationships were found between host health and structure of the skin microbiome; several of which were altered by disease. During periods of shedding, the nature of community assembly varied based on disease state, indicating that disease may alter the effect of shedding, as an acute disturbance, on the host microbiome. Finally, shedding had no significant effect on assemblage richness of the snake microbiome. Consequently, shedding events, which are characterized by the loss of skin and, hence microbial habitat, cannot be considered a source of depletion of the epidermal microbiome in snakes.

Infection with SFD can have detrimental consequences for the host such as increased basal metabolic rate and evaporative water loss (Agugliaro et al., 2019). Ecdysis, or skin shedding, has been implicated as an immune response to SFD infection (Lorch et al., 2015). The control snakes in this study had higher body condition, as predicted by scaled mass index, relative to the inoculated snakes. This suggests that infection with *O. ophiodiicola*, even under food replete conditions, can

lead to depression of host body condition. This is consistent with the physiological costs of the immune response in reptiles, depression of body condition and increased metabolism, which are mediated primarily by elevated corticosterone (Smith et al., 2017). Higher pathogen load, as measured by qPCR assay, was associated with shorter time until death. Consequently, if the pathogen load of an animal increased, mortality was more likely to occur in the near future. It is impossible to conclusively determine that animals died from SFD via the methods utilized here. However, this does suggest that snakes with high or increasing pathogen loads are more likely to be in poor health. Shedding has been proposed as a mechanism for clearing *O. ophiodiicola* based on the observed disappearance of clinical signs after the shed process is complete (Lorch et al., 2015). Shedding in inoculated snakes was correlated with a significant decrease in pathogen load which demonstrates that shedding is an effective host mechanism for reducing pathogen load. These results underscore the suite of interactions that occur between host and pathogen during infection. However, the effects of both host health and disease state on epidermal microbial assemblages must also be considered to develop a comprehensive understanding of the pathology of SFD.

The microbiome is linked to human health, including processes of innate immunity (Grice and Segre 2011), nutrient absorption (Krajmalnik-Brown et al., 2012), and metabolism (Li et al., 2008). Microbial assemblages are also found ubiquitously on the tissues of other animals (Colston and Jackson 2016). In this investigation, control snakes had significantly lower richness of their microbiome compared to inoculated animals. Assemblage richness is sensitive to many factors including disturbance (Compin and Céréghino 2003; Connell 1978), ecological drift

(Gilbert and Levine 2017), productivity (Cornell and Karlson 2000; Gillman and Wright 2006), regional richness (Karlson and Cornell 2002; Srivastava 1999), and landscape heterogeneity (Fahrig et al., 2011; Pollock et al., 1998). In this experiment, the environmental reservoir of microbes (soil) was standardized, which controlled for differences in regional richness. Thus, regional soil richness was unlikely to have a strong impact on local skin richness (Srivastava 1999). Differences in community assembly can result in changes to the structure of microbial assemblages (Stegen et al., 2012). For example, the increased prevalence of drift can increase richness of the lizard microbiome (Lankau et al., 2012). However, in this study, assembly processes were similar between inoculated and control animals. Consequently, suitable explanations for disparate community richness observed here include changes in productivity (Smith 2007), increased landscape heterogeneity in inoculated snakes (Liu et al., 2000), or the effects of disturbance (Dethlefsen et al., 2008). Microbes associated with the epidermis of humans are thought to metabolize host skin excretions, such as urea and lipids (Grice and Segre 2011). The availability of organic compounds on the snake epidermis may be altered by disease (Coates et al., 2014; Park et al., 2013) or host immune response (Drake et al., 2008). Disease mediated changes in the availability of organic compounds could result in decreased community richness via a variety of diversity-productivity relationships (Bernstein et al., 2017; Smith 2007). Heterogeneity of environmental variables is typically positively related to richness due, in part, to increased niche space (Stein et al., 2014) and richness-heterogeneity relationships have been observed among microbial assemblages (Liu et al., 2000). Clinical signs of skin disease are known to generate both distinct microbial assemblages and environmental conditions (Gao et al., 2008; Nakatsuji and Gallo 2019). Consequently, the development of clinical signs

associated with SFD may increase environmental heterogeneity in the epidermal environment. This would explain patterns of elevated richness in inoculated snakes. Finally, disturbance events can directly affect species richness (Armesto and Pickett 1985; Kimbro and Grosholz 2006; Wilson and Tilman 2002). The relationship between disturbance and richness varies depending on the ecological context; however, a widely accepted model relating these variables is the intermediate disturbance hypothesis (Connell 1978). This hypothesis suggests that increasing levels of disturbance will increase species richness until a threshold value is reached (Connell 1978). At which point additional levels of disturbance will decrease species richness (Connell 1978). Higher richness in the inoculated snake microbiome is consistent with models of ecological disturbance, particularly, those that incorporate patch dynamics that may result from clinical signs of infection (Roxburgh et al., 2004).

Chronic disturbance to the host microbiome may occur as a result of host response to SFD, thus altering or disrupting typical host-microbe interactions. In this investigation, control animals displayed a positive relationship between body condition and richness; whereas, snakes inoculated with SFD had a negative relationship between body condition and richness. Feedback mechanisms involved in the coupling of host health with microbiome composition include cell signaling pathways (Knights et al., 2013) and the release of host metabolites (Read and Holmes 2017; Ridlon et al., 2015). Disease can alter typical relationships between host health and the microbiome due to the disruption of host physiology (Mutlu et al., 2012) or stimulation of the host immune response (Chehoud et al., 2013; Grice and Segre 2011). For example, SFD-positive animals in good body condition, may mount an

immune response that has cascading effects on their epidermal microbiome. SFD infected snakes may increase production of antimicrobial skin peptides, thereby excluding colonization of some microbial taxa (Van Hoek 2014), which would explain patterns observed in this study. Conversely, the production of antimicrobial peptides, like many metabolically intensive aspects of the immune system, may be diminished in SFD-positive animals in poor body condition (Navarro et al., 2003). The decreased production of antimicrobial peptides may allow a higher diversity of microbial taxa to colonize the epidermal environment, thus increasing richness (Braff et al., 2005), which is consistent with observations in this study. The mechanisms responsible for generating the positive relationship between host body condition and microbiome richness in control animals are currently unknown. However, these results suggest that richness covaries with host health in SFD-negative individuals. Similar patterns have been observed in frogs, where richness of the epidermal microbiome is positively correlated with host survival following exposure to ranavirus (Harrison et al., 2017). The effects of both host health and disease state on the microbiome demonstrate that disturbance to microbial assemblages resulting from SFD can occur due to disease pathology and host response to infection.

Shedding is a central component of host health and, like body condition, was found to have a significant effect on the host microbiome. Shedding was hypothesized to act as an acute disturbance to the snake microbiome; resulting in primary succession. By definition, this would have required the elimination or significant depletion of microbial taxa from the snake epidermis (Walker and Moral 2011). However, shedding was found to have no effect on Shannon-Weaver diversity or species richness. Structure (β-diversity) of the snake microbiome was altered during

shedding with inoculation status mediating some of its effects. During acute disturbance to the microbiome, such as antibiotic administration, assemblage structure sometimes returns to a pre-disturbance state after a period of several weeks (Dethlefsen et al., 2008; Relman 2012). During the short-term timeseries, inoculation status was found to effect total beta-diversity, turnover, and nestedness of the snake microbiome showing that infection alters the microbiome of diseased snakes; even in the hours immediately following shedding (Allender et al., 2018; Walker et al., 2019). In amphibians, chytridiomycosis is known to modify epidermal microbial assemblages (Jani and Briggs 2014). Alteration to the microbiomes of diseased amphibians is speculated to represent pathogenic dysbiosis, potentially disrupting normal function of the host microbiome and subsequently host physiology (Jani and Briggs 2014; Jiménez and Sommer 2017). Consequently, the maintenance of distinct skin assemblages by inoculated snakes through shedding may have important implications for disease pathology. Over the course of the long-term timeseries, the interaction between inoculation status and shed state was correlated with beta diversity. In particular, differences were found between pre-shed and shed assemblages for both inoculated and control animals. This indicates that shedding rapidly (< 24 hrs) alters the microbiome structure from a pre-disturbance state. This timeline is comparable to the effects of other acute disturbances to the microbiome, such as antibiotic administration (Carlson et al., 2017; shade et al., 2013; Yao et al., 2016). Assemblage turnover was also altered by shed state during the long-term timeseries. Turnover can be conceptualized as the gain and loss of taxa from an assemblage with replacement (Baselga 2010). Consequently, shedding events are associated with the loss of microbial taxa from the microbiome and replacement via colonization of novel taxa. Within macroscopic assemblages, elevated rates of

turnover are associated with disturbance and succession suggesting that shedding is an important factor in structuring the snake microbiome (Shugart and Hett 1973; Shurin 2007).

During shedding, deterministic processes had a greater effect on community assembly in inoculated snakes relative to control snakes. Additionally, during shedding, multivariate dispersion of beta-diversity was greater in inoculated snakes relative to control snakes. Taken together, this suggests that, as a result of shedding, the microbiomes of inoculated snakes become more dissimilar than would be expected due to stochastic processes alone. Traditionally, disturbance events were thought to reduce the impact of priority effects thereby decreasing beta-diversity (Jiang and Patel 2008). However, experimental evidence shows that high disturbance regimes can increase beta-diversity to a greater degree than low disturbance regimes (Hawkins et al., 2015; Séguin et al., 2014). In a similar fashion to the intermediate disturbance hypothesis, quadratic relationships have been found between disturbance, community dissimilarity, and deterministic influences on community assembly (Fraschetti et al., 2001; Lepori and Malmqvist 2009). This pattern is thought to occur when high magnitude disturbance events generate heterogenous environmental filtering on a per-site basis (Hawkins et al., 2015). Therefore, during shedding events, factors likely contributing to increased dissimilarity among inoculated snake microbiomes include host response to infection and fungal pathogenesis. The reptile immune response can be modulated by individual and environmental factors such as season (Hussein et al., 1979; Ridi et al., 1981), temperature (Rios and Zimmerman 2015; Wright and Cooper 1981), hydration (Moeller et al., 2013), maternal diet during development (Itonaga et al., 2011), age (Beck et al., 2017), and stress (Martínez

Silvestre 2014). The immune system is an important factor in structuring the microbiome (Oh et al., 2013; Rakoff-Nahoum et al., 2004; Thaiss et al., 2014). Thus, variation in the host immune response during shedding, a period of high turnover, could lead to heterogenous colonization of taxa in the microbiomes of different snakes (Grice and Segre 2011). Additionally, the degree of fungal pathogenesis and outcome in the form of clinical disease signs vary as disease progresses. Just after shedding, the clinical signs of disease in a given individual may represent a unique heterogenous landscape, in which only a small subset of microbes are able to persist (Gao et al., 2008; Nakatsuji and Gallo 2019). A heterogenous skin landscape could explain higher-than-expected assemblage dissimilarity in inoculated snakes after shedding. In control animals, the acute disturbance of shedding is not compounded with the chronic disturbance of disease. Shedding generated more stochastic community assembly and increased community similarity in control animals compared to inoculated animals. This may occur because the decreased magnitude of disturbance which is generated from shedding alone outweighs priority effects without introducing additional environmental filters (Fraschetti et al., 2001; Symons and Arnott 2014). The differential effects of shedding on the host microbiome may contribute to differences between the microbiomes of diseased and healthy individuals.

In weekly samples of the microbiome, snakes with elevated body condition were found to have distinct microbiome structure. Additionally, chronic disturbance (i.e. inoculation) resulted in the formation of distinct microbial assemblages at equivalent host body conditions. However, in weekly samples of the microbiome, the relative contribution of deterministic and stochastic processes to community assembly

did not vary based on inoculation status. Community structure was significantly different between treatment groups (i.e. an environmental variable), indicating that deterministic assembly processes must be involved in structuring the snake microbiome (Chase and Meyers 2011; Vellend et al., 2014). This is occurs because stochastic assembly processes are by definition not dependent on environmental covariates (Zhou et al., 2013, Zhou and Ning 2017). The Raup-Crick metric only measures the relative strength of assembly processes within a system and not the mechanisms that structure a community (Chase et al., 2011). Assemblages may have deterministic environmental filters of relatively similar strength but resulting from different mechanisms. If this occurs, the Raup-Crick metric would not indicate a difference in assembly because a similar degree of determinism structures both communities (Makhalanyane et al., 2013; Stegen et al., 2012). However, because their environmental filters are generated via different processes, they would possess distinct community structure (Makhalanyane et al., 2013; Stegen et al., 2012). Within the snake microbiome, chronic disturbance did not increase the relative strength of deterministic assembly processes but did generate distinct assemblage structure. This suggests that different environmental filters of similar strength affect the epidermal microbiome based on disease status (Goldford et al., 2018; Weiher and Keddy 1995). The ability of disease to alter environmental filters is consistent with the prediction that disturbance should effect taxa based on their ability to tolerate disturbed conditions (Berga et al., 2012; Marxsen et al., 2010). Previous work has also found a high degree of endogenous environmental filtering associated with the epidermis of herpetofauna lending credence to this hypothesis (Kueneman et al., 2014; Walke et al., 2014; Walker et al., 2019). Furthermore, this suggests that changes in the

microbiome of infected snakes are due to disease processes rather than just changes in the nature of community assembly.

Given the growing threat of emerging infectious disease to wildlife in the 21st century, developing an increased understanding of disease is crucial to generating effective conservation strategies. Additionally, wildlife diseases are an active area of research for understanding of host-pathogen-microbiome interactions. Non-human systems allow for theoretical developments to microbial ecology under conditions which do not exist in the context of mammalian hosts. This study has shown that the application of disturbance ecology can be useful in interpreting the effects of both pathogen and host behaviors on the microbiome. Chronic disturbance to the host microbiome was found to occur as a result of disease; causing alterations to alpha and beta diversity. The mechanisms underlying this disturbance appear to be linked to the host immune response. Acute disturbance to the host microbiome was found to occur as a result of shedding; causing alterations to beta diversity. Additionally, assembly was found to be more deterministic during shedding events in inoculated snakes. This demonstrates that compounding of disturbance events in diseased individuals can lead to changes in the structure and assembly of the microbiome. Unique aspects of the biology of a host species, such as shedding, must be considered when attempting to determine factors that may influence the structure and assembly of the microbiome through time. Furthermore, this system demonstrates that, like macroscopic assemblages, microbiomes are sensitive to changes in the environment and react predictably to perturbation. Additional work is needed to determine if disruption of the host microbiome by SFD is directly associated with deteriorating host health, such as diminished body condition.

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APPENDICES

Appendix A: Figures

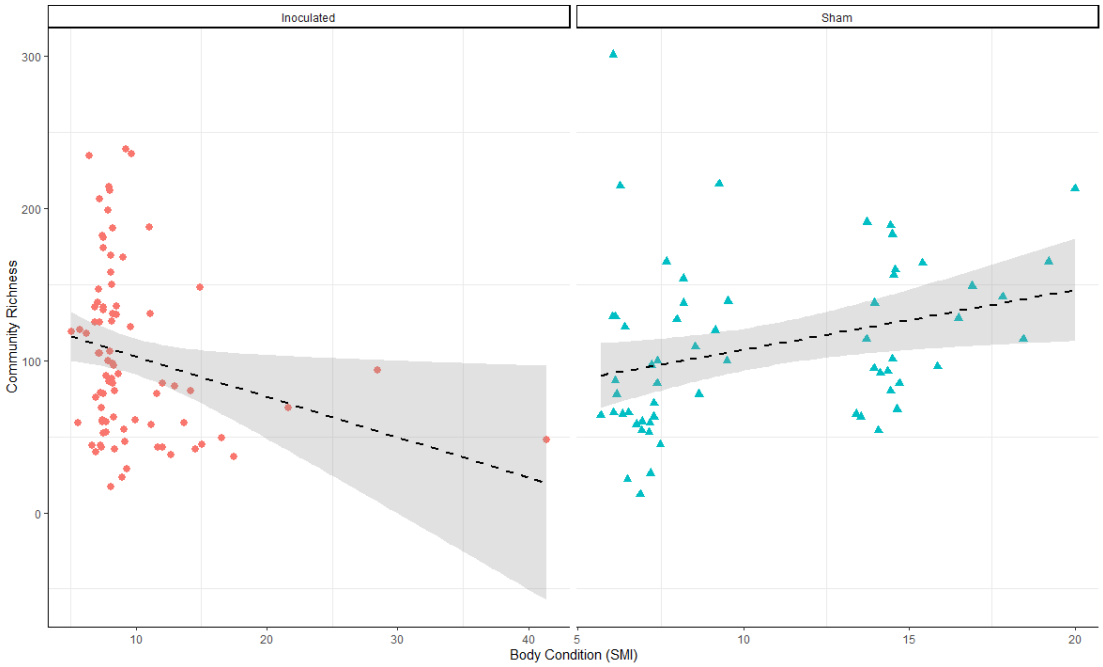

Figure 1. Richness Related to Body Condition and Experimental Treatment. Assemblage richness of the microbiome as a function of host body condition (SMI). The plot is separated into inoculated individuals (red) and control individuals (blue) as relationships between richness and body condition were different based on inoculation status. Relationships were modeled using a linear mixed-effects model.

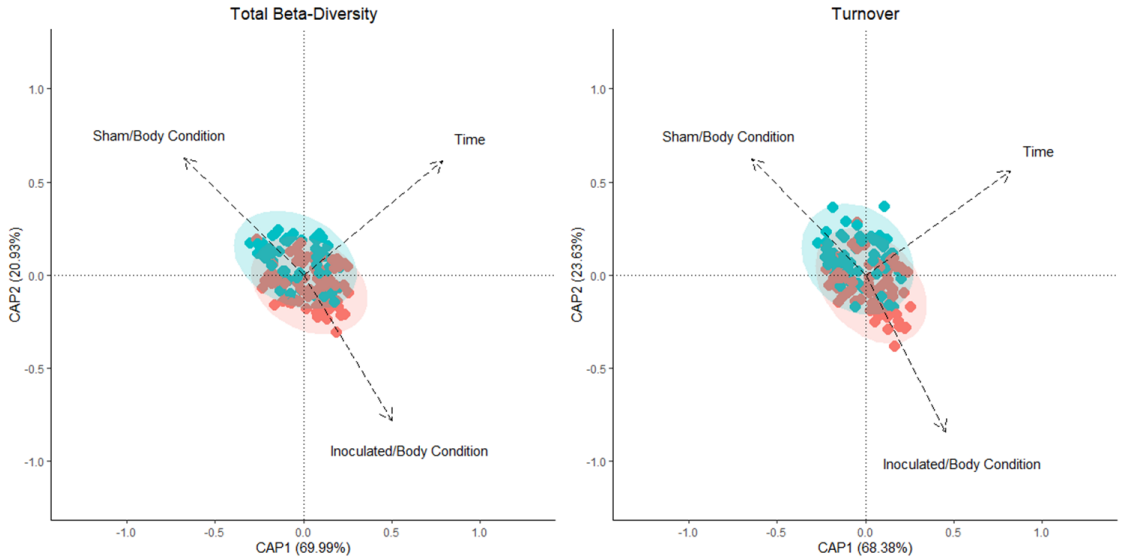

Figure 2. Total & Turnover Diversity Altered by Body Condition and Inoculation. Distance based redundancy analysis and constrained ordination of total beta-diversity and turnover component of beta-diversity from weekly sampling dataset. Red points are associated with inoculated individuals and blue points are associated with control individuals. Elevated body condition correlated with distinct assemblage structures based on inoculation status.

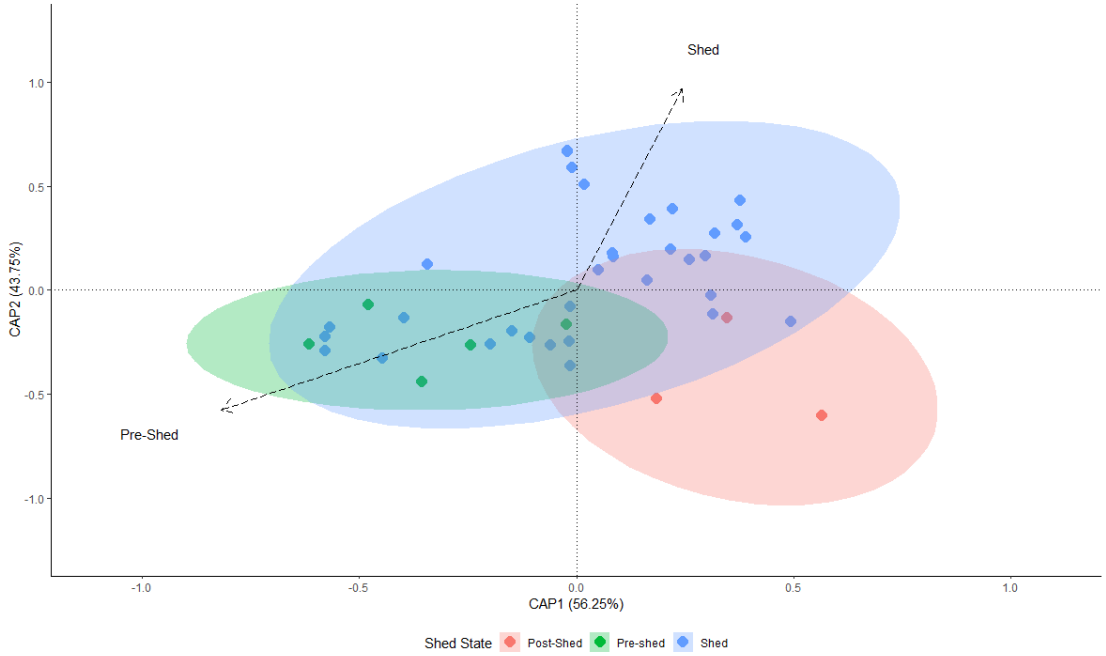

Figure 3. Turnover Diversity is Altered by Acute Disturbance Generated by Shedding. Distance based redundancy analysis and constrained ordination indicated that assemblage turnover differed before, during and after the process of skin shedding. This suggests that shedding significantly modifies the structure of snake microbiome from a pre-shed state; particularly with regard to the gain and loss of taxa from the system with replacement.

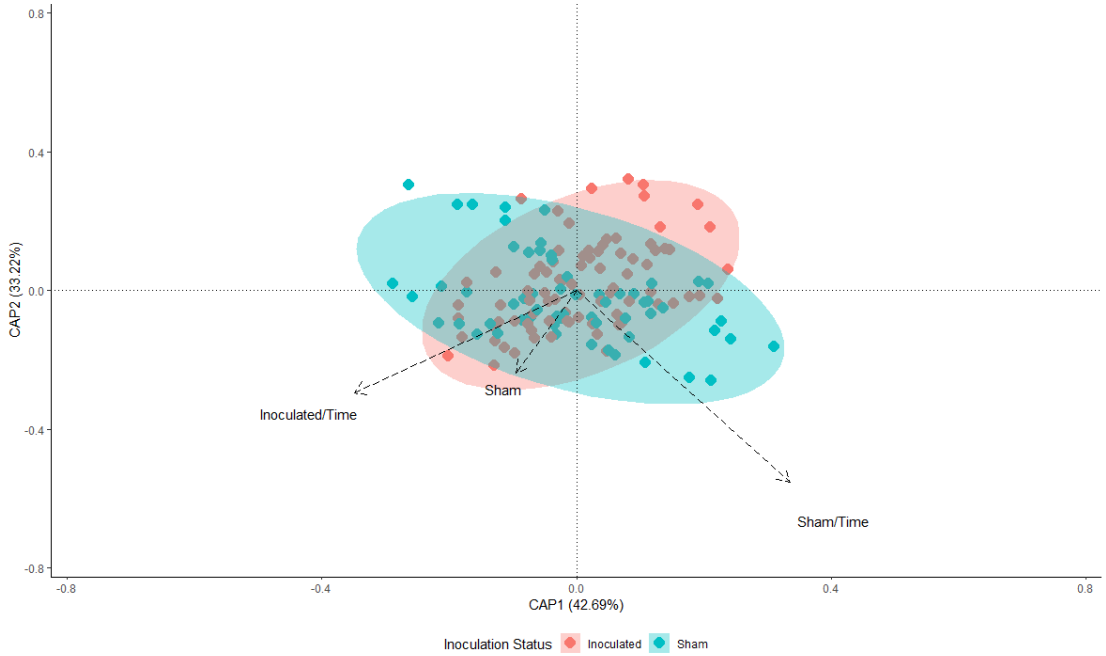

Figure 4. Chronic Disturbance Generated by Disease Doesn't Alter Assembly. Distance based redundancy analysis and constrained ordination of the Raup-Crick metric generated from weekly sampling timeseries. Ellipse size indicates that inoculated samples do not deviate from the null expectation more than control samples. This suggests the nature of community assembly does not differ based on disease status.

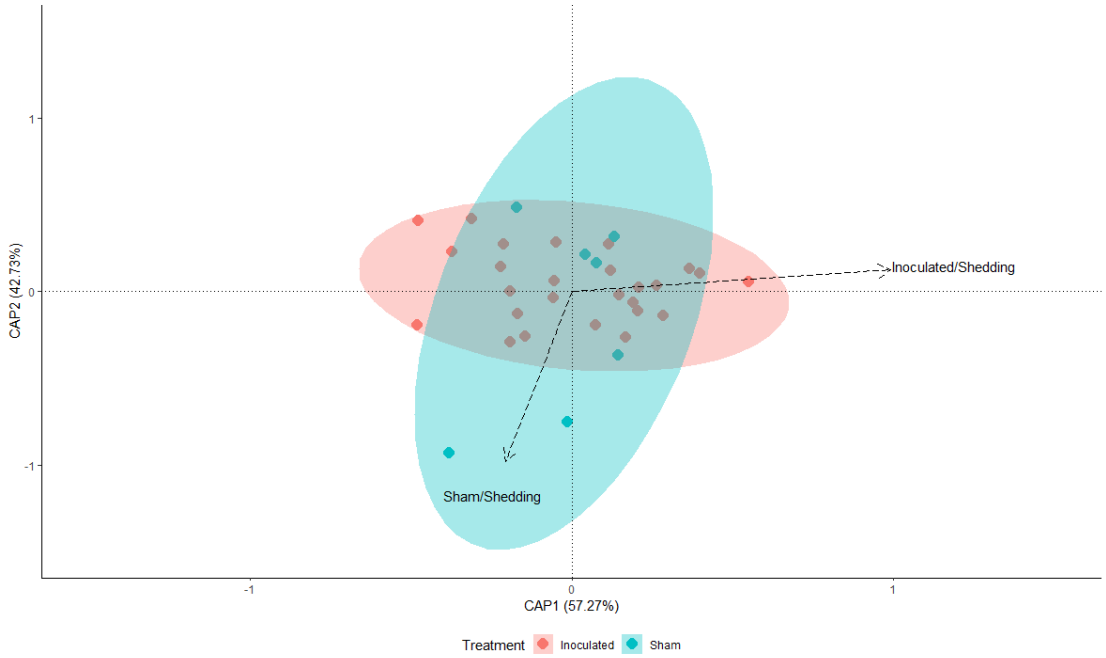

Figure 5. Acute Disturbance Generated by Shedding Alters Assembly in Diseased Snakes. Distance based redundancy analysis and constrained ordination of the Raup-Crick metric generated from short-term shedding timeseries. Ellipse size indicates that inoculated snakes deviate from the null expectation more than control samples. This suggests that inoculated snakes have more deterministic community assembly during shedding than control snakes.

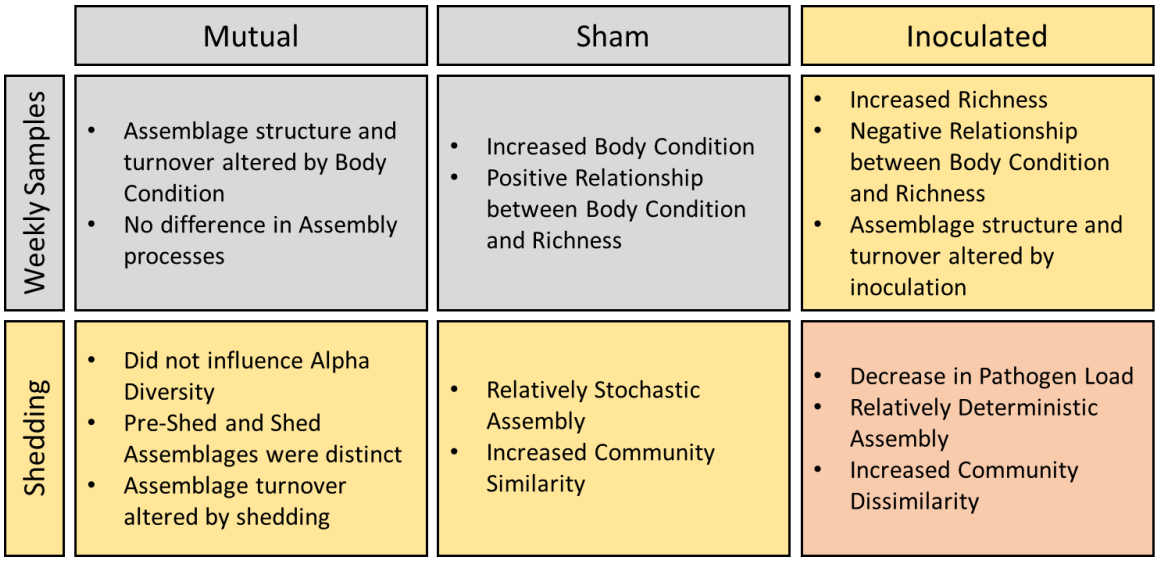

Figure 6. Both Shedding and Inoculation have Significant Effects on the Microbiome. Synthesis of main effects of inoculation (chronic disturbance) and shedding (acute disturbance) on snake health and the microbiome. Potential effects of disturbance are highlighted in yellow with compounding effects of disturbance highlighted in orange. Note that results shared between treatment groups are located in the "Mutual" column.

Appendix B: Tables

Table 1. Overview of Experimental Animals. Data displayed in this table includes initial morphometrics, capture location, and mortality date. All experimental animals were Northern Watersnakes (*Nerodia sipedon*). The inoculated treatment group was exposed to the fungal pathogen *Ophidiomyces Ophiodiicola*.

Animal ID	Capture Location	Capture date	Treatment Group	Initial SVL (cm)	Initial Weight (g)	Clinical Survival	Mortality date
e1	Cheatham	4/21/2019	Inoculated	15.8	9.38	No	6/3/2019
e2	Cheatham	4/28/2019	Inoculated	17.2	5.99	No	6/10/2019
e3	Cheatham	5/1/2019	Inoculated	19.1	8.96	No	7/29/2019
e4	Putnam	5/7/2019	Inoculated	19.2	9.6	No	6/19/2019
e5	Putnam	5/16/2019	Inoculated	19.7	3.88	No	6/29/2019
e6	Putnam	5/19/2019	Inoculated	18.5	4.06	Yes	8/21/2019
e7	Putnam	5/19/2019	Inoculated	21.4	5.98	Yes	8/21/2019
e8	Putnam	5/22/2019	Inoculated	18.2	3.7	Yes	8/21/2019
e9	Rutherford	5/22/2019	Inoculated	35.5	27.33	Yes	8/21/2019
e10	Rutherford	5/24/2019	Inoculated	21.5	5.41	Yes	8/21/2019
e11	Rutherford	5/24/2019	Inoculated	44.6	44.63	No	6/18/2019
c1	Cheatham	4/21/2019	control	18.1	3.43	No	6/12/2019
c2	Cheatham	5/1/2019	control	17.8	3.25	No	6/24/2019
c5	Putnam	5/16/2019	control	34.5	16.5	No	8/14/2019
c7	Putnam	5/19/2019	control	18.3	7.33	Yes	8/21/2019
c9	Rutherford	5/24/2019	control	23.5	13.68	No	7/20/2019
c10	Rutherford	5/24/2019	control	23.6	14.91	Yes	8/21/2019
c11	Rutherford	5/24/2019	control	23.2	18	Yes	8/21/2019

IACUC APPROVAL

IACUC
INSTITUTIONAL ANIMAL CARE and USE COMMITEE
Office of Research Compliance,
010A Sam Ingram Building,
2269 Middle Tennessee Blvd
Murfreesboro, TN 37129

MIDDLE TENNESSEE STATE UNIVERSITY

IACUCN006: FCR PROTOCOL APPROVAL NOTICE

Thursday, May 14, 2020

Principal Investigator	**Donald Walker**
Co-Investigator(s):	NONE
Investigator Email(s):	*donald.walker@mtsu.edu*
Department/Unit:	Biology
Protocol ID:	**19-3012**
Protocol Title:	***Effects of snake fungal disease on epidermal microbiome community dynamics***

Dear Investigator(s),

The MTSU Institutional Animal Care and Use Committee has reviewed the animal use proposal identified above under the ***Full Committee Review (FCR) mechanism***. The IACUC met on 3/22/2019 to determine if your proposal meets the requirements for approval. The Committee determined through a unanimous vote that this protocol meets the guidelines for approval in accordance with PHS policy but required additional clarifications and revisions as described in the review notice served to you on 03/28/2019. The IACUC also voted to review your revisions by an accelerated DMR mechanism. Your revisions were received on 04/04/2019 and an accelerated DMR was conducted on the revised protocol. Based on the DMR, the IACHC has approved this protocol for the proposed animal use. A summary of the IACUC action(s) and other particulars of this this protocol are tabulated below:

IACUC Action	**APPROVED for one year**	
Date of Expiration	4/30/2021	
Number of Animals	14 (FOURTEEN)	
Approved Species	**Nerodia sipedon**	
Category	☐ **Teaching**	☒ **Research**
Subclassifications	☐ Classroom	☒ Laboratory ☒ Field Research ☐ Field Study
	☐ Laboratory	☒ Handling/Manipulation ☐ Observation
	Comment: NONE	
Approved Site(s)	Animals captured/obtained from wild-life management areas in Tennessee. The animals will be housed and analyzed in SCI 1170 Rooms G & F	
Restrictions	Must comply with all FCR requirements;	
Comments	NONE	

This approval is effective for three (3) years from the date of this notice. This protocol expires on 4/30/2022 The investigator(s) MUST file a Progress Report annually regarding the status of this study. Refer to the schedule for Continuing Review shown below; NO REMINDERS WILL

BE SENT. A continuation request (progress report) must be approved by the IACUC prior to 4/30/2021 for this protocol to be active for its full term. Once a protocol has expired, it cannot be continued and the investigators must request a fresh protocol.

Continuing Review Schedule:

Reporting Period	Requisition Deadline	IACUC Comments
First year report	3/31/2020	A continuing review (CR) was conducted on this protocol. The IACUC determined by a unanimous vote on 05/12/2020 that this protocol can continue for an additional year
Second year report	3/31/2021	Yet to be completed
Final report	3/31/2022	Yet to be completed

Post-approval Amendments:

Date	*Amendment*	*IACUC Notes*
06/22/2019	Animal feed changed to live fish	Accelerated DMR
07/25/2019	Emma Phipps (ejp3e: CITI8128034) and and Kylie Bowe (kcm4f: CITI8144413) are approved to work in this protocol	Accelerated DMR

Post-approval Actions:

Date	*Amendment*	*IACUC Notes*
02/27/2020	Minor discrepancy in the animal number was notified by the PI. The IACUC reviewed the update through FCR and determined that it was a genuine human error and dismissed any potential sanctions	FCR 02/27/2020

MTSU Policy defines an investigator as someone who has contact with live or dead animals for research or teaching purposes. Anyone meeting this definition must be listed on your protocol and must complete appropriate training through the CITI program. Addition of investigators requires submission of an Addendum request to the Office of Research Compliance.

The IACUC must be notified of any proposed protocol changes prior to their implementation. Unanticipated harms to subjects or adverse events must be reported within 48 hours to the Office of Compliance at (615) 494-8918 and by email – compliance@mtsu.edu.

All records pertaining to the animal care be retained by the MTSU faculty in charge for at least three (3) years AFTER the study is completed. Be advised that all IACUC approved protocols are subject to audit at any time and all animal facilities are subject to inspections at least biannually. Furthermore, IACUC reserves the right to change, revoke or modify this approval without prior notice.

Sincerely,

Compliance Office
(On behalf of IACUC)
Middle Tennessee State University