THE MYCOBIOME AND SKIN CHEMISTRY OF BAT WINGS IN RELATION TO WHITE-NOSE SYNDROME

A dissertation submitted to the Committee of Graduate Studies

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the faculty of Arts and Science

TRENT UNIVERSITY

Peterborough, Ontario, Canada

Environmental and Life Sciences Graduate Program

September 2022

ABSTRACT

The mycobiome and skin chemistry of bat wings in relation to white-nose syndrome

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White-nose syndrome (WNS) is a skin disease of bats caused by the fungus *Pseudogymnoascus destructans* (*Pd*) that damages flight membranes during hibernation and can lead to death. The disease causes mortality of multiple bat species in eastern North America and is spreading into western North America. Future impacts of WNS on naïve bat populations are unknown. Variation in host susceptibility occurs among and within species, but mechanisms driving this variation are unclear. Multiple studies have characterized immunological responses to WNS, but skin physiology as a barrier to pathogens is understudied. The unique ability of *Pd* to actively penetrate the normal, intact skin of its mammalian host makes WNS an interesting study system to understand skin defenses. Aspects of the mammalian skin environment that can influence disease susceptibility include pH, sebaceous lipids, and microbiomes. I found skin mycobiomes of WNS-susceptible species had significantly lower alpha diversity and abundance compared to bat species resistant to *Pd* infection. Using these data, I predicted that most naïve bat species in western North America will be susceptible to WNS based on the low diversity of their skin mycobiomes. Some fungi isolated from bat wings inhibited *Pd* growth *in vitro*, but only under specific salinity and pH conditions, suggesting the microenvironment on wings can influence microbial interactions and potentially WNS-susceptibility. I measured the wing-skin pH of bats in eastern Canada and found that *Eptesicus fuscus* (WNS-tolerant) had more acidic skin than *M. lucifugus* (WNS-susceptible). Differences in sebum quantity and composition among and within mammalian species may help explain variation in skin disease susceptibility

and the composition of skin microbiomes. This is due to the antimicrobial properties of sebum and the use of sebum as a nutrition source by microbes. Outcomes of this work further our understanding of inter- and intra-specific differences among bat species and individuals in skin mycobiomes and physiology, which may contribute to variation in WNS-susceptibility. Future research should focus on characterizing the physical and chemical landscape of skin as this is essential for understanding mechanisms structuring skin microbial assemblages and skin disease susceptibility in wildlife.

KEYWORDS: skin microbiome, mycobiome, skin pH, white-nose syndrome, bats, *Myotis*, *Pseudogymnoascus*, yeast, fungi, cave, sebum, mammal, sebaceous, disease susceptibility

DEDICATION

In memory of my father and fellow scientist, Cornelius H. Vanderwolf

ACKNOWLEDGEMENTS

I completed the initial research for my thesis at the University of Wisconsin under the supervision of Drs. Jeffrey Lorch, Tony Goldberg, and David Blehert. I am grateful for the opportunities they provided and for enabling the production of chapters two and three of my thesis. I completed the second half of my thesis at Trent University and I am particularly grateful to Dr. Christina Davy for making this possible and for her unwavering support. I would like to express my deepest appreciation to Dr. Christopher Kyle, Dr. Donald McAlpine, and Dr. Michael Donaldson for their supervision and advice. Many thanks to members of the Kyle, Davy, and Goldberg labs for their support in and out of the lab and field. I am grateful to the Natural Sciences and Engineering Research Council of Canada for financial support. Thanks to Dr. Karen Thompson, Dr. Diana Northup, and chair Dr. Jeff Bowman for an interesting discussion during my defense.

This work would not have been possible without the many field biologists who helped coordinate sampling efforts and collected samples, including B. Abel, Chris Anderson, Linda Anger, Anne Ballmann, Laura Beard, B. Bybee, Allen Calvert, Shelly Colatskie, Katie O'Connor, Keith Day, Krysta Demere, R Doening, Tony Elliott, Julia Ersan, Jennifer Fox, Nate Fuller, Kyle George, Katie Gillies, Mary Gilmore, Catherine Haase, Lindsey Heffernan, Carl Herzog, Catherine Hoose, Daryl Howell, Anthony Kerwin, Cori Lausen and Wildlife Conservation Society Canada, Liam McGuire, Angie McIntire, Eathan McIntyre, Sarah Olson, Jennifer Redell, Gabriel Antonio Reyes, David Riggs, Blake Sasse, Michael Scafini, Nick Sharp, Kirk Silas, Craig Stihler, Shawn Thomas, Abby Tobin, Greg Turner, Ted Weller and the Redwood Hoary Bat Crew, Paul White, Jason Williams, Rebecca Williams, R. Winton, and Michelle Verant. I am also profoundly grateful to the many people who helped with field work:

Yan Bourque, Lucy Brown, Lisa Browning, Mark Browning, Laura Bruce, David Christie, Maddie Empey, Brock Fenton, Janet Greenhorn, Adam Grottoli, Lesley Hale, Hannah Hamblin, Allie Hjort, Ryan Holt, Lauren Hooton, Howard Huynh, Sophia Konieczka, Jennifer Larkin, Tessa McBurney, Emily McNaughton, Kevin Quirion-Poirier, Carrie Sadowski, Laura Scott, Jordi Segers, Véronique St-Hilaire, Andrew Sullivan, Olivier Trudel, Dylan White, and Val Von Zuben. I thank Doerte Doepfer, Erica Newton, Lewis Campbell, and James Paterson for providing statistical advice and assistance with R wrangling. Katie Schmidt, Daniel Taylor, and Rick Lankau provided technical assistance with laboratory work. Many thanks to Tina Egstad, Elizabeth Bohuski, Brenda Zier, Natalie Dayal, Karin McMullen, and Kyle George for their camaraderie, assistance, and advice in the lab. Last, but certainly not least, special thanks to my family and DF McAlpine for seeing me through.

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LIST OF ABBREVIATIONS

BHI	brain heart infusion
CFU	colony forming units
DNA	deoxyribonucleic acid
DTM	dermatophyte test medium
ITS	internal transcribed spacer
LNA	Leeming and Notman agar
OTU	operational taxonomic unit
PBST	phosphate buffered saline
Pd	*Pseudogymnoascus destructans*
PERMANOVA	non-parametric permutational multivariate analysis of variance
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscopy
SD	sabouraud agar
WNS	white-nose syndrome
YM	yeast morphology medium

CHAPTER 1

General introduction

Infectious diseases are a major concern in conservation biology and appear to be increasing in prevalence due to climate change, pollution, habitat destruction/alteration, genetic bottlenecks, and globalization (Altizer *et al.*, 2003; Brearley *et al.*, 2013; Pisano *et al.*, 2019; Cohen *et al.*, 2020). Increased exposure to domestic and feral animals, environmental degradation, and biodiversity loss are all associated with increased pathogen exposure and disease risk, and these problems are likely to increase in severity and scope in the future (Daszak *et al.*, 2000, 2001; Cunningham *et al.*, 2017). Understanding mechanisms by which some species or populations resist disease can inform management strategies for threatened species, yet the underlying drivers leading to varied disease outcomes are poorly understood.

Immune responses are an important driver, but it is also critical to understand mechanisms by which pathogens enter the body and how the body resists pathogen establishment before immune responses are activated. Pathogens can enter the body through mucosal surfaces in the gastrointestinal, urogenital, and respiratory tracts (van Ginkel *et al.*, 2000), as well as the skin. Skin represents the primary interface between a host and the environment and the first line of defense against pathogens.

Skin is a complex physical and chemical landscape, and defenses against microbial invasion include the combined effects of desiccation, epidermal desquamation, acidic pH, nutrient limitations, commensal microbes, and antibodies (Harder et al. 2013; Naik et al. 2012). Physiological and morphological differences across the integument, such as presence of hair and glands, can cause variations in temperature, pH, moisture, nutrient availability, and the

composition of antimicrobial peptides and lipids (Kearney *et al.*, 1984; Grice *et al.*, 2009; Findley *et al.*, 2013; Schommer and Gallo, 2013). This effectively creates diverse microhabitats across the surface of the skin that affect the density and diversity of microbial colonization, including pathogen establishment and growth (Kearney *et al.*, 1984; Harder *et al.*, 2013). For example, enzymes secreted by human fungal skin pathogens, such as *Candida* sp., have temperature and pH optima in order to function effectively (Tronchin *et al.*, 2008). Microbial colonization is not restricted to the surface of the skin as substantial populations are also associated with skin appendages, especially sebaceous follicles, where sebum represents one of the major potential nutrient sources for microbes (Harder et al. 2013; Kearney et al. 1984; Naik et al. 2012). Networks of microbe–microbe interactions on the skin surface may govern host inflammation and disease outcomes in a strain- and context-dependent manner (Chen et al. 2018).

Skin microbiomes comprise a diversity of fungi, bacteria, and viruses (Byrd *et al.*, 2018). Yeasts (fungi that can reproduce by budding) are more common than filamentous fungi on human skin, such as species of *Cryptococcus*, *Malassezia*, and *Candida*, and the abundance and diversity of these yeasts varies among body sites and individuals depending on physiological conditions on the skin surface (Byrd et al. 2018; Huffnagle and Noverr 2013). In humans, the microbial diversity of sebaceous skin sites, such as the face and upper body, differs from dry sites such as the forearm and buttock; bacterial diversity is less on sebaceous skin sites while yeasts show the reverse trend (Sanmiguel and Grice, 2015). Increasing the alkalinity of human skin has been associated with increased bacterial counts (Dikstein and Zlotogorski, 1994), and products that decrease skin pH are used on dogs to combat skin infections (Matousek et al.

2003). The effect of skin physiology on skin microbiome composition and function in wild mammals has yet to be studied.

Diverse mycobiome (i.e., that portion of a microbial community, or microbiome, comprised of fungi) patterns are associated with decreased prevalence or severity of various disease processes in humans and can play a role as preventive or therapeutic agents (Cui et al. 2013). For instance, high diversity of the skin mycobiome is correlated with decreased severity of skin disorders such as atopic dermatitis and psoriasis (Zhang *et al.*, 2011; Kong and Morris, 2017). Skin microbiomes can have direct antimicrobial properties against pathogens, modify the local skin environment to inhibit pathogen growth, either suppress or activate the host immune system, and outcompete pathogens for space and resources on the skin surface (Cui *et al.*, 2013; Byrd *et al.*, 2018). In wildlife, examples of the role skin microbiomes play in defense against pathogens include studies focused on microbes that can inhibit growth of the amphibian-infecting chytrid fungus *Batrachochytrium dendrobatidis* that causes chytridiomycosis, a skin disease (Harris *et al.*, 2009; Becker and Harris, 2010). Most microbiome studies related to chytridiomycosis have focused on bacteria, but a recent study found the cutaneous mycobiome may contribute more to defense against *B. dendrobatidis* than resident bacteria (Kearns *et al.*, 2017). However, the role of the mycobiome in health and disease is an emerging field of research that has lagged behind research on the bacterial microbiome (Cui, et al. 2013; Huffnagle and Noverr 2013; Kong and Morris 2017).

A major skin disease of wildlife is white-nose syndrome (WNS), a cutaneous infection of bats caused by the fungal pathogen *Pseudogymnoascus destructans* (*Pd*) that damages flight membranes during hibernation and can lead to starvation, dehydration, and death (Lorch *et al.*, 2011a; Cryan *et al.*, 2013). Since its initial detection in North America in 2006, WNS has killed

more than 6.5 million bats (U.S. Fish and Wildlife Service, 2012; Cheng *et al.*, 2021), making it one of the most devastating wildlife diseases ever documented. In 2016, *Pd* was documented in western North America for the first time (Lorch *et al.*, 2016), and several western bat species have subsequently been diagnosed with clinical WNS or found with *Pd* on their skin (White-nose syndrome response team, 2020). However, the extent of future impacts of the disease on populations of western bat species remains unclear. Three species of bats that were abundant prior to the arrival of WNS have experienced such massive population declines in eastern North America due to the disease that they are now listed as threatened or endangered in the U.S. or Canada (Frick et al. 2016). The Little Brown Bat (*Myotis lucifugus*), Northern Long-eared Bat (*M. septentrionalis*), and Tricolored Bat (*Perimyotis subflavus*) have experienced massive population declines due to WNS, while other species [e.g., Big Brown Bat (*Eptesicus fuscus*) and Eastern Small-footed Bat (*M. leibii*)] appear to be more tolerant of the infection and have persisted (Frank et al. 2014; Langwig et al. 2012; Turner et al. 2011). Still other species of hibernating bats [e.g., Townsend's Big-eared Bat (*Corynorhinus townsendii*) and Rafinesque's Big-eared Bat (*C. rafinesquii*)] appear to be entirely resistant to the disease, as *Pd* has been detected on their skin without clinical WNS (Bernard et al. 2015, 2017; Turner et al. 2011). Variation in host susceptibility has also been documented within species, as some colonies of *M. lucifugus* have persisted after more than a decade of exposure to *Pd* (Reichard *et al.*, 2014).

The invasive nature of *Pd* contrasts with the behavior of typical dermatophytes of mammals, such as *Trichophyton, Microsporum*, and *Epidermophyton* spp., which are superficial parasites that cause skin diseases such as ringworm and athletes' foot (Meteyer *et al.*, 2022). *Pseudogymnoascus destructans* hyphae form cup-like epidermal erosions and ulcers in the wing membrane with involvement of underlying connective tissue, and hair follicles, sebaceous and

apocrine glands, connective tissue, blood and lymphatic vessels, and elastin and muscle fibers of normal wing tissue are replaced with hyphae (Meteyer *et al.*, 2009, 2022; Cryan *et al.*, 2010). Most fungi that can cause pathology in animals exist primarily as saprobes and are opportunistic pathogens that do not require an animal host (Berbee, 2001). *Pseudogymnoascus destructans* is unique among known mammal-associated cutaneous fungi in its ability to actively penetrate the normal, intact skin of its host. This makes WNS an interesting study system to better understand mammalian skin defenses against microbial pathogens.

Bats represent more than 20% of mammalian species and play an important role in wildlife communities and ecosystems (Kunz and Fenton, 2003). Insectivorous bats consume many insects each night, some of which are economically-important pests (Boyles *et al.*, 2011; Kunz *et al.*, 2011). Of the 45 bat species in Canada and the United States, 21 species regularly use caves during the winter or summer (Pierson, 1998) and therefore may be exposed to *Pd*. Bats that live in temperate regions are all insectivorous species that must cope with seasonally fluctuating temperatures and food supplies by one of two methods: hibernation or migration. Bats do not hoard food and year-round residents must hibernate when food is absent for long periods, so bats accumulate body fat deposits in late summer and early autumn prior to hibernation (Ewing *et al.*, 1970). Female bats form maternity colonies from May to July in order to give birth and raise their pups, while males roost separately (Kunz and Fenton, 2003).

Physical and chemical features of bat wings likely affect skin disease establishment and progression, but little baseline information is available. Previous studies of bat wings have mainly focused on aerodynamic parameters such as wing shape, elastin and collagen fiber patterns, and muscular structure (Holbrook and Odland, 1978; Swartz *et al.*, 1996; Swartz and Konow, 2015). In bat wings sweat glands have been reported as either absent (Sokolov, 1982;

Makanya and Mortola, 2007) or exclusively apocrine (Sisk, 1957; Cortese and Nicoll, 1970). Bat wings have sebaceous glands which vary in abundance by species (Cortese and Nicoll, 1970; Sokolov, 1982; Yin *et al.*, 2011), although Sokolov (1982) reported that sebaceous glands cease to function in bats during winter hibernation.

Multiple host traits have been hypothesized as contributing to variable impacts of WNS, including body size, length of hibernation period, physiology, immune defenses, hibernation behavior, and skin microbial assemblages (Warnecke *et al.*, 2012; Frank *et al.*, 2014, 2016; Field *et al.*, 2015; Hayman *et al.*, 2017; Lemieux-Labonté *et al.*, 2017; Moore *et al.*, 2018). The composition of integumentary lipids, which are known to have anti-microbial properties but can also be a nutrition source for microbes, differs among bat species such as *M. lucifugus* (WNS-susceptible) and *E. fuscus* (WNS-tolerant) (Muñoz-Garcia *et al.*, 2012; Pannkuk *et al.*, 2012; Ben-hamo *et al.*, 2016; Ingala *et al.*, 2017). Some of the free fatty acids that differ between the wing integument of *M. lucifugus* and *E. fuscus* inhibit *Pd in vivo* (Frank *et al.*, 2016; Ingala *et al.*, 2017), but results are contradictory (Neville, 2017). The bacterial microbiome may also play a protective role in bats that survive WNS (Hoyt *et al.*, 2015; Lemieux-Labonté *et al.*, 2017).

Physiological changes to the skin resulting from WNS may impact skin microbial diversity by changing nutrient availability and compromising the skin's ability to prevent microbial growth. The impact of WNS on the skin microbiome appears to be species-specific, with lower bacterial diversity on *M. lucifugus* when *Pd* was present, but with no significant effect on *E. fuscus* or *P. subflavus* (Lemieux-Labonté *et al.*, 2017, 2020; Ange-Stark *et al.*, 2019). This may partially reflect species-specific differences in skin physiology such as lipid composition and pH (Pannkuk *et al.*, 2012), as well as differing responses to WNS. Some

bacterial taxa are enriched on *Pd*-positive bats while other bacteria are enriched on *Pd*-negative bats (Lemieux-Labonté *et al.*, 2017; Ange-Stark *et al.*, 2019; Grisnik *et al.*, 2020).

Bat skin mycobiomes vary with both species and site (Johnson et al. 2013; Vanderwolf et al. 2013b; Vanderwolf et al. 2015, 2016), though species may be more important than site in determining yeast composition (Njus, 2014). Skin mycobiomes were more diverse on WNS-negative bats (Johnson *et al.*, 2013), although (Vanderwolf *et al.*, 2016) found no difference in skin mycobiome composition between WNS-negative and positive bats. Although a variety of fungi are present on bat skin (Larcher *et al.*, 2003; Voyron *et al.*, 2011; Vanderwolf *et al.*, 2013b, 2016; Borda *et al.*, 2014; Holz *et al.*, 2018; Fenster *et al.*, 2019; Ogorek *et al.*, 2020; Furtado *et al.*, 2021), it is usually unclear which species can grow there. The dermatophyte *Trichophyton redellii* grows on bat skin and can cause superficial infections (Lorch *et al.*, 2015). Other skin diseases, such as various forms of dermatitis, have also been documented in bats, some of which are caused by fungi (Simpson *et al.*, 2013; Goodnight, 2015; McAlpine *et al.*, 2016; Fountain *et al.*, 2017; Tamayo *et al.*, 2021).

Research Objectives

The objectives for the research presented in this thesis were: 1) determine if skin mycobiome composition and abundance varied with species-level WNS-susceptibility, and 2) examine aspects of skin physiology, namely pH and sebum, that may influence microbial composition and microbial interactions on the skin, as well as disease outcomes. To address these objectives, in chapter two I compared skin mycobiome characteristics of ten bat species that differ in susceptibility to WNS sampled across ten eastern U.S. states using culture-dependent techniques (Vanderwolf *et al.*, 2021a). Culture-dependent techniques were chosen to

ensure only viable microbes were detected, as these microbes are more likely to be commensals. I hypothesized that culturable constituents of bat skin fungal assemblages differ based on host WNS-susceptibility. In chapter three I compared skin mycobiome characteristics of thirteen bat species sampled across eleven states and one province in western North America, and therefore of unknown WNS-susceptibility, to mycobiome characteristics of eastern bats of known WNS-susceptibility (Vanderwolf *et al.*, 2021c). I did this to predict WNS-susceptibility of western bat species. I tested the most common fungi to determine if they inhibited *Pd* growth *in vitro*. In chapter four I characterized the skin pH of bat wings to determine to determine how it differs with species, season, body part, sex, age-class, geographic location, and pH of roosting substrates. (Vanderwolf *et al.*, 2021b). Inter- and intra-specific or seasonal variation in bat skin pH may partially explain corresponding variation in cutaneous microbiomes and responses to pathogens such as *Pd*. For chapter five I had planned pre-COVID-19 to determine the relationship between the diversity and composition of bat skin mycobiomes to variation among and within individuals and species in skin pH. I found in chapters two and three that some individual bats had particularly high fungal diversity and abundance (Vanderwolf *et al.*, 2021ac), which may be due to their skin physiology. In chapter four I found that skin pH varied among species and individuals within species (Vanderwolf *et al.*, 2021b). The objective was to determine if skin pH correlates with the diversity and composition of microbiomes on bat skin. If skin pH is a factor driving microbiome diversity, there will be high intra-individual variation in microbiomes among body sites that differ in pH. If individual exposure histories drive microbiome diversity, there will be low intra-individual variation and high inter-individual variation, regardless of skin pH. I predicted that bats with low wing pH will have lower bacterial diversity but higher yeast diversity compared to bats with high wing pH. I measured skin pH on

four different parts of flight membranes of *Myotis lucifugus* and *Eptesicus fuscus* in southern Ontario, Canada, and collected swabs from the same body parts. Swabs were to be processed using next-generation sequencing to determine the composition of the microbiome (bacteria and fungi). Unfortunately, due to lockdowns during the COVID-19 pandemic the lab work for this project was delayed to the extent that it could not be completed within the timeline for my doctoral research. Therefore, for chapter five I synthesized current knowledge of sebum function in wild mammals in relation to skin diseases and skin microbiomes. Sebum is an important component of the skin barrier and contributes to skin defenses against both biotic and abiotic stressors. Greater knowledge of skin physiology will facilitate *in vitro* experiments studying potential outcomes of microbial interactions on the skin surface.

The thesis is presented as a compilation of four manuscripts, each representing an individual chapter. It concludes with a general discussion in which the implications of these works and future directions are discussed. Overall, the manuscripts are linked as the first two examine skin mycobiome variation among bat species while the last two examine two factors, namely sebum and skin pH, that may influence the composition and abundance of skin mycobiomes, skin disease occurrence and progression, and microbe-microbe interactions on the skin. This body of work advanced knowledge of skin mycobiomes and skin pH among bat species in North America in relation to WNS-susceptibility. This thesis also synthesized current knowledge on skin pH and sebum among wild mammals with the intent of fostering future research on the role of skin in maintaining health and preventing disease in wildlife.

CHAPTER 2

PREFACE

Title: Skin fungal assemblages of bats vary based on susceptibility to white-nose syndrome

Authors: Karen J. Vanderwolf, Lewis J. Campbell, Tony L. Goldberg, David S. Blehert, and Jeffrey M. Lorch.

Reference: Published in *The ISME Journal* (2021), **15**: 909-920
https://www.nature.com/articles/s41396-020-00821-w
2020 Impact Factor: 10.3

Contributions: T.L.G., D.S.B, and J.M.L. conceived, directed, and obtained funding for the research presented in this study. J.M.L. and K.J.V. cultured the fungi and performed PCR. J.M.L. performed the phylogenetic analyses on *Debaryomyces*, *Cutaneotrichosporon*, and *Leucosporidium* and created Fig. 2.S3 and Table 2.S5. J.M.L. and K.J.V. conceived methods for the inhibition assays. K.J.V. performed the statistical analysis, the inhibition assays, the scanning electron microscopy, and created Table 2.1, 2.2, 2.S1, 2.S2, 2.S3, 2.S4, 2.S6, 2.S7, 2.S8, and Fig. 2.2, 2.3, 2.S1, 2.S2. L.J.C. provided statistical advice and created Fig. 2.1. K.J.V drafted the manuscript and made revisions according to reviewer's recommendations. J.M.L. edited the initial draft. All authors edited the manuscript prior to submission and after subsequent revision according to reviewer's recommendations. C.D. agreed that the contributions of K.J.V. are as stated, above.

CHAPTER 2

Skin fungal assemblages of bats vary based on susceptibility to white-nose syndrome

Published in *The ISME Journal* (2021), **15**: 909-920

https://www.nature.com/articles/s41396-020-00821-w

ABSTRACT

Microbial skin assemblages, including fungal communities, can influence host resistance to infectious diseases. The diversity-invasibility hypothesis predicts that high-diversity communities are less easily invaded than species-poor communities, and thus diverse microbial communities may prevent pathogens from colonizing a host. To explore the hypothesis that host fungal communities mediate resistance to infection by fungal pathogens, we investigated characteristics of bat skin fungal communities as they relate to susceptibility to the emerging disease white-nose syndrome (WNS). Using a culture-based approach, we compared skin fungal assemblage characteristics of 10 bat species that differ in susceptibility to WNS across 10 eastern U.S. states. The fungal assemblages on WNS-susceptible bat species had significantly lower alpha diversity and abundance compared to WNS-resistant species. Overall fungal assemblage structure did not vary based on WNS-susceptibility, but several yeast species were differentially abundant on WNS-resistant bat species. One yeast species inhibited *Pseudogymnoascus destructans* (*Pd*), the causative agent on WNS, *in vitro* under certain conditions, suggesting a possible role in host protection. Further exploration of interactions between *Pd* and constituents

of skin fungal assemblages may prove useful for predicting susceptibility of bat populations to WNS and for developing effective mitigation strategies.

INTRODUCTION

A potential microbial invader into an ecosystem must overcome barriers to invasion including abiotic (e.g. pH, temperature, and salinity) and biotic (e.g., competition, antagonism, and predation) resistance (Mallon *et al.*, 2015). The diversity-invasibility hypothesis predicts that high-diversity communities are less easily invaded than species-poor communities (Case, 1990; Kennedy *et al.*, 2002; Mallon *et al.*, 2015). For example, reduction of skin microflora on amphibians increases host susceptibility to and mortality from the chytrid fungus *Batrachochytrium dendrobatidis* (Harris *et al.*, 2009; Becker and Harris, 2010). Diversity is not the only characteristic of the microbiome that may influence disease susceptibility. Specific components of microbiome may also inhibit pathogen invasion through production of antimicrobial compounds, stimulation or training of the host immune system, and occupation of adherence sites and metabolic niches (Harder *et al.*, 2013; Chen *et al.*, 2018). Thus, the presence of certain microorganisms rather than the overall diversity or community structure may be responsible for host resistance.

Although most work on skin microbiomes focus on bacterial communities, the role of skin fungal assemblages in health and disease is an emerging field (Cui *et al.*, 2013; Huffnagle and Noverr, 2013; Kong and Morris, 2017). For example, a recent study found cutaneous fungal assemblages may contribute even more to defense against *B. dendrobatidis* than do bacteria (Kearns *et al.*, 2017). Diverse skin fungal assemblage patterns are associated with various diseases in humans and can play a role as preventive or therapeutic agents (Cui *et al.*, 2013). For

instance, the severity of skin disorders such as atopic dermatitis and psoriasis are inversely correlated with the diversity of skin fungal assemblages (Kong and Morris, 2017). Commensal microorganisms in humans, including fungi, play a role in appropriately tuning immune activity to ensure efficient responses to pathogens while limiting responses directed toward host tissues and innocuous agents such as allergens (Hooper *et al.*, 2012; Chen *et al.*, 2018).

The role of the fungal skin microbiome in susceptibility to and severity of emerging fungal diseases in wildlife is an active area of investigation, with previous work focusing on amphibian chytridiomycosis and snake fungal disease (Kearns *et al.*, 2017; Allender *et al.*, 2018). However, examination of how the fungal skin microbiome mediates white-nose syndrome (WNS) in bats has not been studied. White-nose syndrome, a cutaneous fungal infection of hibernating bats caused by *Pseudogymnoascus destructans* (*Pd*), was introduced to North America from Eurasia (Lorch *et al.*, 2011a; Frick *et al.*, 2016). The fungal pathogen damages wing membranes, resulting in physiological disruptions that can lead to death (Cryan *et al.*, 2013). Since its initial detection in North America in 2006, WNS has killed more than 6.5 million bats (U.S. Fish and Wildlife Service, 2012), making it one of the most devastating wildlife diseases ever documented. Some species of bats have experienced catastrophic population declines due to WNS and are now listed as endangered (Solari, 2018).

Variation in host susceptibility to WNS has been documented within and between bat species. For example, the little brown bat (*Myotis lucifugus*), northern long-eared bat (*My. septentrionalis*), and tricolored bat (*Perimyotis subflavus*) have experienced massive population declines due to WNS, while other species [e.g., big brown bat (*Eptesicus fuscus*), eastern small-footed bat (*My. leibii*), and Indiana bat (*My. sodalis*)] appear to resist infection by *Pd* (have lower pathogen burdens) and have persisted, even in areas where *Pd* appears to be established in

hibernacula (Turner *et al.*, 2011; Langwig *et al.*, 2012; Frank *et al.*, 2014; Frick *et al.*, 2017). Still other species of hibernating bats [e.g., Townsend's big-eared bat (*Corynorhinus townsendii*) and Rafinesque's big-eared bat (*Co. rafinesquii*)] appear to be unaffected by the disease, in that they display no lesions diagnostic for infection despite exposure to *Pd* (Turner *et al.*, 2011; Bernard *et al.*, 2015, 2017; White-nose syndrome response team, 2020). Variation in host susceptibility has also been documented within species, for example some colonies of *My. lucifugus* in New York state are persisting after more than a decade of exposure to *Pd* (Reichard *et al.*, 2014). In all, 11 bat species have been documented with WNS in North America, and *Pd* has been detected on an additional six species without clinical signs of WNS (Turner *et al.*, 2011; Bernard *et al.*, 2015, 2017; White-nose syndrome response team, 2020). Multiple host traits have been hypothesized as determining WNS-susceptibility including body size, length of hibernation period, physio-chemistry (e.g. sebaceous lipid composition), immune defenses, hibernation behavior, and skin microbial assemblages (Warnecke *et al.*, 2012; Frank *et al.*, 2014, 2016; Field *et al.*, 2015; Hayman *et al.*, 2017; Lemieux-Labonté *et al.*, 2017; Moore *et al.*, 2018). However, the precise mechanisms of WNS-resistance remain unknown.

We examined skin fungal assemblage characteristics of the wings of 10 bat species across the eastern United States. We hypothesized that culturable constituents of bat skin fungal assemblages differ by species-level WNS-susceptibility. Due to complex nature of a host's microbiome and the various ways in which it can confer resistance to disease, no one analysis is sufficient for testing this hypothesis. Therefore, we tested two sub-hypotheses to address our larger question. For our first sub-hypothesis, we predicted that WNS-resistant bat species would have higher fungal diversity and abundance on their wings compared to WNS-susceptible species (consistent with the pre-existing diversity-invasibility hypothesis). For our second sub-

hypothesis, we predicted that we would detect fungal species on WNS-resistant bats that were rare or absent from susceptible bats, and that these fungal species would directly inhibit *Pd* growth *in vitro*.

METHODS

Sampling

Field sampling was approved by the U.S. Geological Survey National Wildlife Health Center Institutional Animal Care and Use Committee (Protocols #EP140212 and #EP081124-A2). Samples were collected from 25 hibernacula in the eastern USA (10 states) from January to March, 2014 – 2017 (Table 2.1; Figure 2.1). Sampling targeted hibernating bats during winter because *Pd* primarily grows on bats during hibernation.

Categorizing bat species into WNS-susceptibility groups is an area of active debate because disease processes act on a context-dependent continuum (Davy *et al.*, 2018). For this study, we classified *My. lucifugus*, *My. septentrionalis,* and *Per. subflavus* as WNS-susceptible because these species have experienced WNS-associated population declines of over 70% in multiple hibernacula (Turner *et al.*, 2011; Thogmartin *et al.*, 2012). We classified *Co. rafinesquii* and Virginia big-eared bat (*Co. townsendii virginianus*) as WNS-impervious as these species have not been documented with *Pd* infections, despite repeated detection of *Pd* on their wing skin (indicating exposure) (Bernard *et al.*, 2015, 2017). We classified species as WNS-resistant if they are documented to develop *Pd* infections, but do not exhibit large-scale mortality as a direct result of WNS. Under this classification, *E. fuscus* and *My. leibii* were categorized as WNS-resistant as they have exhibited <50% WNS-associated declines within *Pd*-infected populations (Turner *et al.*, 2011). Similarly, population declines related to WNS have not been documented

in southeastern myotis (*My. austroriparius*) and gray bat (*My. grisescens*) despite confirmation of WNS in these species (Turner *et al.*, 2011; Powers *et al.*, 2016; Bernard and McCracken, 2017; US Geological Survey, 2017). *Myotis sodalis* was also classified as WNS-resistant because declines in *Pd*-infected *My. sodalis* populations have been variable. At some locations, *My. sodalis* colonies have experienced >70% declines after the detection of WNS, while at other sites declines have been <50% (or the colony has even increased) post-WNS (Turner *et al.*, 2011; Thogmartin *et al.*, 2012; Ingersoll *et al.*, 2013; Powers *et al.*, 2015). Additionally, fungal loads of *Pd* on the skin of *My. sodalis* are more consistent with those found on WNS-resistant species (Frick *et al.*, 2017). Some individual bats within a resistant species may develop WNS severe enough to cause mortality, while other individuals have low pathogen loads. We emphasize that our classifications are made at the species level and are not necessarily valid for all individuals within a species as we did not track the fate of individual bats in this study.

We swabbed wings by rolling a sterile Pur-Wraps® polyester-tipped swab (Puritan Medical Products Company LLC, Guilford, Maine, USA), pre-moistened with 150 µl sterile nuclease-free water, three times across the ventral plagiopatagium (wing membrane connecting the hindlimb and forelimb). We stored swabs in individual, sterile, 1.5 ml microcentrifuge tubes at 4 ºC. Swabs were stored from 1 to 21 days with a mean of 3.9 ± 3 days. We changed nitrile gloves between handling individual bats to prevent cross-contamination.

Fungal Culture and Identification

We streaked each swab five times, discretely, across three different media: sabouraud dextrose agar with chloramphenicol and gentamicin (SD; BD Diagnostic Systems, Sparks, Maryland, USA), dermatophyte test medium (DTM) with chloramphenicol, cycloheximide

(added to inhibit fast-growing saprophytic fungi), and gentamicin (prepared in-house), and modified Leeming and Notman agar (LNA; prepared in-house (Lorch *et al.*, 2018)). Plates were sealed with laboratory film (Bemis Flexible Packaging, Neenah, Wisconsin) and incubated in darkness at 7°C (to approximate typical conditions in hibernacula) for 2 months. We checked plates weekly and isolated morphologically unique fungal colonies, including *Pd*, in pure culture. We counted the number of colonies of each morphotype on each plate weekly to determine colony forming units (CFUs) until confluent growth precluded accurate counts. The final CFU count for each morphotype on each bat was calculated by summing the total CFUs of each morphotype on the three media per each individual bat.

We identified pure cultures by analyzing the full-length internal transcribed spacer (ITS) region of the fungal rRNA gene (Lorch *et al.*, 2015). Sequences were collapsed into representative operational taxonomic units (OTUs) using USEARCH (Edgar, 2010) with a 97% similarity threshold (O'Brien *et al.*, 2005). We applied a 99% similarity threshold for the genus *Debaromyces* because of minimal genetic variation exhibited in the ITS region among *Debaromyces* species (Martorell *et al.*, 2005). We assigned taxonomy to sequences in R, using the assigntaxonomy function (DADA2 package) (Callahan *et al.*, 2016) with UNITE (Kõljalg *et al.*, 2013; Community, 2017). Some sequences were not identified to genus using UNITE, and we compared these to NCBI's Genbank database using BLAST(Altschul *et al.*, 1990). We manually generated a community matrix of annotated OTUs and their CFUs for each bat.

Except where noted, we removed *Pd* from the dataset for statistical analyses to focus on skin fungal assemblage characteristics associated with WNS-susceptibility rather than differences caused by the pathogen. We also excluded fungal OTUs that were only isolated from one bat, as these are likely transient species rather than commensals.

Statistical Analyses

We performed analyses in R (R Core Team, 2020). We calculated the Shannon diversity index (hereafter, Shannon Index) of fungi on each bat using the diversity function in the vegan package (Oksanen *et al.*, 2018). To test our first sub-hypothesis that skin fungal assemblage diversity is related to WNS-susceptibility group, we constructed a Gaussian zero-inflated model with Shannon Index as the response variable and bat species (9 level factor), site (23 level factor with New York sites excluded), and WNS-susceptibility group (3 level factor) as explanatory variables (package glmmTMB) (Brooks *et al.*, 2017). We included the number of days swabs were stored, year of collection, month of collection, state (broad spatial scale), and day-of-year swabs were collected in supplemental models to examine which explanatory variables were predictive of variation. We used the function AICtab (package bbmle) (Bolker and Team, 2017) to compare model Akaike information criteria (AIC) values. As yeasts are important components of the cutaneous mycobiome of mammals (Mason *et al.*, 1996; Byrd *et al.*, 2018), we repeated this analysis using yeasts-only Shannon Index, fungal abundance (CFU), and yeast abundance as the response variable in separate analyses. We determined the optimum model family using AIC for abundance response variables. We determined the best data transformation with the transformTukey function (package rcompanion) (Mangiafico, 2019) for each response variable.

To determine if wing fungal assemblage composition varied among WNS-susceptibility groups, bat species, and sites, we implemented a non-parametric permutational multivariate analysis of variance (PERMANOVA) on abundance based (CFU) Bray-Curtis dissimilarity coefficients using the function ADONIS (vegan (Oksanen *et al.*, 2018)). As Bray-Curtis dissimilarity values cannot be calculated for samples that have no composition, we first removed

individual bats with no cultured fungi, individuals from which only *Pd* was cultured, and bats from which the only fungus cultured was the single representative of that OTU within our dataset (n=4 *Co. townsendii virginianus*, n=3 *Co. rafinesquii*, n=1 *E. fuscus*, n=1 *My. leibii*, n=47 *My. lucifugus*, n=14 *My. septentrionalis*, and n=64 *Per. subflavus*). We ran PERMANOVA for 1000 iterations, and report R^2 values when the variable enters the model last.

To test our second sub-hypothesis, we used DESeq2 (version 1.10.1, alpha = 0.05) to identify OTUs that were differentially abundant among WNS-susceptibility groups, correcting for multiple pairwise comparisons using Benjamini-Hochberg adjustment (Love *et al.*, 2014), and including bats with no cultured fungi (DESeq2 allows non-balanced datasets). As differential abundance analysis does not account for within-group consistency, we also performed an indicator species analysis using the multipatt function (indicspecies package) (De Cáceres and Legendre, 2009). We report OTUs with an indicator statistic above 0.4 (Lemieux-Labonté *et al.*, 2017).

To determine if the abundance of *Pd* on a bat, as determined by our culture-dependent results, affected skin fungal assemblages, we constructed zero-inflated models using either fungal abundance (truncated negative binomial) or alpha diversity (represented by Shannon Index; Gaussian distribution) as the response variable, *Pd* abundance (CFU counts; explanatory variable), and bat species and site (random effects). The models with Shannon Index did not converge, so we determined the best data transformation with the transformTukey function (package rcompanion) (Mangiafico, 2019) for both the response (Shannon Index, lambda=0.4) and explanatory variables (*Pd* abundance, lambda=0.225). To examine whether *Pd* abundance influenced the fungal community composition of each bat (beta diversity), a PERMANOVA was run as described above. These analyses were only run with bat species on which *Pd* was cultured

from at least one individual bat, but included all individuals sampled within those species (*My. lucifugus, My. septentrionalis, My. sodalis,* and *Per. subflavus*).

Mycobiomes on WNS-Resistant vs. Susceptible Populations of *Myotis lucifugus*

We sampled colonies of *My. lucifugus* from two sites in New York during winter 2014-2015 as described above. These bat colonies have persisted despite the ongoing presence of *Pd* since 2006 (Reichard *et al.*, 2014). We compared samples from these resistant bat colonies to samples collected from *My. lucifugus* colonies farther west (Wisconsin and Kentucky) that were naïve to WNS at the time of sampling and thus considered susceptible. We used zero-inflated models with either Shannon Index (Gaussian), fungal abundance (truncated negative binomial), or yeast abundance (truncated negative binomial) as the response variable, and WNS-susceptibility group (resistant vs. susceptible) as the sole explanatory variable with site (7-level factor) as a random effect. We also performed a differential abundance analysis as described above.

Inhibition Assays

We screened five yeast OTUs that were differentially enriched on WNS-resistant or impervious bats for *Pd*-antagonism by spore-germination and growth-inhibition assays. The production of toxins by fungi varies with environmental conditions (Marquina *et al.*, 2001). Therefore, different types of media, pH, and salt conditions were tested, including SD (pH 5.6), brain heart infusion (BHI, pH 7.4), BHI with 10% sheep blood (pH 7.4), yeast morphology (YM) medium with the pH adjusted to either 4.5, 5.0, or 7.0 with 0.1 M citrate-phosphate buffer, and YM supplemented with 6% (w/v) NaCl at pH 5.0. We supplemented media at pH 4.5 with

increased agar (2% w/v) to ensure solidification. To determine whether *Pd* would grow on medium with increased NaCl, we supplemented YM at pH 5.0 with NaCl at half percent intervals, inoculated with pure cultures of *Pd*, and incubated at 7 ºC for 2 months. *Pseudogymnoascus destructans* grew on YM at both pH 4.5 and 5.0 (no NaCl supplementation). We saw visible *Pd* growth on YM pH 5.0 supplemented with 0.5% to 2.0% NaCl after 2 weeks incubation, 2.5% to 4.0% NaCl after 1 month, and 4.5% to 6.0% NaCl after 2 months. All yeast strains used in the assays also grew under these conditions.

For inhibition assays, we harvested *Pd* conidia (ATCC MYA-4855) from 3-month old cultures as described by Lorch *et al*. (Lorch *et al.*, 2011a) and enumerated conidia using a hemocytometer. We spread 150 µl of conidial suspension containing two million conidia onto agar medium. We placed six pre-sterilized Whatman #1 filter paper discs equidistant from one another on each plate. We harvested yeasts from 6-day old cultures grown on SD at 7 ºC by scraping cells off agar surfaces with sterile loops and suspending them in sterile phosphate buffered saline (PBST) containing 0.5% Tween 20. We enumerated yeast cells as described above and 8 µl suspensions each containing 500,000 cells were pipetted directly onto individual filter-paper discs. We tested each yeast-strain in triplicate, incubating all plates in the dark at 7 ºC. Negative controls were discs treated with PBST only and positive controls were discs containing voriconazole (30 µg; Sensi-disc; Becton, Dickinson, & Co., Franklin Lakes, New Jersey). We checked plates daily for the first week to assess inhibition of *Pd* germination near the discs, and then weekly for 2 months or until *Pd* growth had covered the entire agar surface. We measured zones of inhibition around the discs to the nearest mm after 2 weeks of incubation.

RESULTS

The Skin Mycobiome Differs Between Susceptible and Resistant Bat Species

We processed 398 swabs from 10 bat species sampled across 10 states (Table 2.1). Fungi were cultured from 86.9% (346) of swabs, representing 137 fungal morphotypes of 80 genera. We found that 20.9% of OTUs were cultured from a single individual bat (Table 2.S1). The most commonly cultured fungi were *Pd* (from 32.4% of bats), *Debaryomyces hansenii* (29.1%), *Cutaneotrichosporon moniliiforme* (17.3%), *Malassezia vespertilionis* (14.6%; newly described during this study (Lorch *et al.*, 2018)), two additional unassigned *Debaryomyces* species [hereafter referred to as *Debaryomyces* sp. 1 (14.3%) and *Debaryomyces* sp. 3 (12.3%)], and *Cladosporium delicatulum* (8.5%). Yeasts (including dimorphic fungi) comprised 32.1% of detected OTUs.

Yeasts, when present, often had high CFU counts compared to filamentous fungi. This is shown in Figure 2.2 as patterns among bat species and sites for overall fungal abundance (A) are virtually identical to yeast abundance (C). In sites where multiple bat species were sampled, such as AL1, AL2, AL3, KY1, MO1, and WI7 (Figure 2.1, 2.2), WNS-resistant species, such as *My. grisescens* and *E. fuscus*, always had higher fungal and yeast abundance than WNS-susceptible species. WNS-impervious species had low fungal and yeast abundance at all sites (Figure 2.2 A&C). Although filamentous fungi were generally present in low abundance, their diversity was higher than yeast diversity on some bats. This is reflected in Figure 2.2 where bat species with low fungal abundance (A), such as *My. leibii* and *Corynorhinus* spp., had a higher mean Shannon Index (B) compared with bat species with high fungal abundance, such as *My. austroriparius*. Conversely, some bat species had high abundance of one or two OTUs, but low overall diversity as reflected by the Shannon Index (e.g., *My. austroriparius*). WNS-susceptible species had

higher diversity of filamentous fungi (Figure 2.2B) compared to yeast diversity (Figure 2.2D). Fungal abundance was similar among the three bat species in the WNS-susceptible group and between the two impervious *Corynorhinus* spp. (Figure 2.S1 A&B).

Fungal Diversity Differs Among Bat Species

The best model explaining differences in skin fungal diversity (Shannon Index as dependent variable) among individuals included bat species and site (Table 2.S2). Some bat species, such as *Per. subflavus* and *Co. townsendii virginianus*, had significantly lower Shannon Index compared to other bat species such as *E. fuscus* and *My. grisescens* (Table 2.S3). When bat species and site were included as random variables and WNS-susceptibility group was the sole explanatory variable, WNS-impervious (estimate= 0.1703, std error= 0.1094, $p<0.001$) and WNS-susceptible (estimate=0.1244, std error=0.0782, $p<0.001$) species had significantly lower Shannon Indices than WNS-resistant species (estimate=0.5988, std error=0.0600, $p<0.001$) in the conditional model. None of the factors explained variance in the zero-inflation model (estimate= -23.77, std error= 13427.55, $p=0.999$; i.e. there was no pattern among groups, bat species, or sites for which individuals would have a Shannon Index of zero).

Yeast-only Diversity Differs Among Bat Species

The best model with yeast-only Shannon Indices as the dependent variable included bat species and month of collection (Table 2.S2). This model indicated that some bat species, such as *E. fuscus*, *My. grisescens*, and *My. austroriparius*, had significantly higher yeast Shannon Indices than other bat species such as *Per. subflavus* (Table 2.S3). When bat species and site were included as random variables and WNS-susceptibility group was the sole explanatory variable,

WNS-impervious (estimate= 0.0938, std error= 0.0918, p<0.001) and WNS-susceptible (estimate=0, std error=0.0681, p<0.001) species had significantly lower Shannon Indices than WNS-resistant species (estimate=0.4883, std error=0.0509, p<0.001) in the conditional model. None of the factors explained variance in the zero-inflation model (estimate= -25.97, std error= 45955.42, p=1).

Fungal Abundance Differs Among Bat Species

The best models with fungal abundance (CFU counts; response variable) all included bat species (Table 2.S2). The best model (bat species and day-of-year swabs were collected) indicated that *My. grisescens*, *My. austroriparius*, and *My. sodalis* had significantly higher fungal abundance than other bat species (Table 2.S3). When bat species and site were included as random variables and WNS-susceptibility group was the sole explanatory variable, WNS-impervious (estimate= 1.4299, std error= 0.4333, p= 0.0065) and WNS-susceptible (estimate= 1.0361, std error= 0.3632, p<0.001) species had lower fungal abundance compared to WNS-resistant species (estimate= 2.6087, std error= 0.2395, p<0.001) in the conditional model.

Yeast-only Abundance Differs Among Bat Species

The best models with yeast abundance (response variable) all included bat species (Table 2.S2). The best model (bat species and day-of-year swabs were collected) indicated that *My. grisescens*, *My. austroriparius*, and *My. sodalis* had significantly higher yeast abundance than other bat species (Table 2.S3). When bat species and site were included as random variables and WNS-susceptibility group was the sole explanatory variable, WNS-susceptible (estimate= 21.25, std error= 74.84, p=0.0013) and WNS-impervious (estimate= 23.25, std error= 86.32, p=0.0057)

species had significantly lower fungal abundance compared to WNS-resistant species (estimate= 262.09, std error= 48.54, $p<0.001$).

Fungal Composition Differs Among Bat Species

Skin fungal assemblage composition was significantly associated with both bat species (pseudo-$F_{5,207}$=2.833 R^2=0.0386, p=0.001) and site of collection (pseudo-$F_{18,207}$=2.725, R^2=0.1335, p=0.001). White-nose syndrome susceptibility group, month of collection, year of collection, number of days swabs were stored, state, and day of year swabs were collected were not significant predictors of skin fungal assemblage composition (these variables were removed because they did not improve the model). Six OTUs (all yeast) were differentially abundant among WNS-susceptibility groups and identified as indicator species (Figure 2.3).

Relationship Between *Pd* and the Skin Mycobiome

Pd was only isolated from four bat species: from 5.1% of *My. septentrionalis* (n=39), 53.6% of *My. sodalis* (n=28), 49.4% of *Per. subflauvs* (n=79), 91.8% of *My. lucifugus* sampled in New York (n=61), and from 32.6% of *My. lucifugus* sampled elsewhere (n=43; Table 2.S4). When present, *Pd* was abundant (high CFU counts) on *My. lucifugus*, but had low abundance on the only WNS-resistant species from which *Pd* was cultured during this study, *My. sodalis* (Table 2.S4). Neither Shannon Index nor fungal abundance significantly varied with *Pd* abundance in either the conditional model (estimate=0.2692, std error=0.0194, p=0.495 for Shannon Index; estimate= -0.0178 on log scale, std error= 0.0172, p=0.302) or zero-inflation model (estimate= -25.17, std error=31587.79, p=0.999 for Shannon Index; estimate=0.0006, std error= 0.0012, p=0.583 for fungal abundance). The composition of bat skin fungal assemblages

was predicted by both bat species (pseudo-$F_{1,127}$=4.8693, R^2=0.0287, p=0.001) and site (pseudo-$F_{13,127}$=2.2349, R^2=0.1713, p=0.001), but not *Pd* abundance (pseudo-$F_{1,127}$=1.4216, R^2=0.0084, p=0.086).

Mycobiomes of WNS-Resistant versus WNS-Susceptible Populations of *Myotis lucifugus*

Resistant *My. lucifugus* in New York had lower fungal abundance (estimate= -16.934 on log scale, std error= 0.6056, p<0.001), lower yeast abundance (estimate= -14.8541, std error= 0.7492, p<0.001), and more individuals with zero yeast colonies (estimate= 0.5521, std error=0.2036, p=0.0067) compared to susceptible *My. lucifugus* in Wisconsin and Kentucky. However, resistant *My. lucifugus* colonies did not have more individuals with zero fungal colonies (estimate= -0.1929, std error=0.197, p=0.328) or different Shannon Indices (estimate= 0.0484, std error= 0.08554, p=0.1627) than susceptible *My. lucifugus. Malassezia vespertilionis* was identified as differentially abundant on susceptible as compared to resistant *My. lucifugus* (log2Change= -5.897, p<0.001).

Skin Mycobiome Constituents from WNS-resistant Bats Inhibit *Pd* Under Certain Conditions

Of the yeast strains tested, two isolates of *Cu. moniliiforme* inhibited *Pd in vitro* under certain conditions (Table 2.2). On YM at pH 5.0 with 6% NaCl medium, *Pd* grew slowly with contorted hyphae (germination of spores was limited and first seen after 4 weeks, but inhibition results could only be assessed 7 weeks post-inoculation), indicating *Pd* was likely stressed. The diameter of inhibition rings decreased over time and completely disappeared on all plates, including positive controls, after 4 weeks of incubation. The only exception was the positive

control YM pH 5 with 6% NaCl, on which very limited germination of *Pd* was first observed after 10 weeks of incubation.

DISCUSSION

We hypothesized that the skin microbiomes of bats would vary based on WNS-susceptibility group. More specifically, we tested the sub-hypothesis that that WNS-resistant bat species would have more diverse and abundant skin fungal assemblages as compared to susceptible bat species. Although resistant species did have higher Shannon Indices and fungal abundance compared to susceptible species, bat species that are impervious to *Pd* infection did not. Njus (2014) also found that bat species with low WNS-associated mortality (*Co. townsendii* and *E. fuscus*) were disproportionally colonized by yeasts, particularly *Debaryomyces* spp., compared to species with high WNS-associated mortality (*My. lucifugus*). The lack of culturable fungi on WNS-susceptible and impervious species was supported by the absence of non-*Pd* fungi observed on *My. lucifugus*, *Per. subflavus*, and *Co. rafinesquii* wings using SEM (Appendix 1). We also found that susceptible *My. lucifugus* colonies had higher fungal abundance compared to resistant colonies. Therefore, mechanisms other than skin fungal assemblages contribute to infection outcomes in WNS-impervious *Corynorhinus* spp. and persisting colonies of WNS-resistant *My. lucifugus*. Disease resistance is often multifactorial, and combinations of host genetic, physiological, and behavioral characteristics also likely influence WNS susceptibility (Medzhitov *et al.*, 2012).

Distinguishing transient fungi from commensals is a common challenge for skin microbial assemblage studies (Kramer *et al.*, 2015; Nash *et al.*, 2017). Many genera we isolated (e.g., *Penicillium, Cladosporium,* non-*Pd Pseudogymnoascus*, and *Mortierella*) are commonly

isolated from cave environments and considered saprotrophs that are unlikely to colonize bat skin (Johnson *et al.*, 2013; Lorch *et al.*, 2013; Vanderwolf *et al.*, 2013b, 2016). In contrast, the high abundance of certain yeast taxa (e.g., *Debaryomyces, Cutaneotrichosporon, Leucosporidium*, and *Ma. vespertilionis*), combined with our SEM observations of yeasts budding on the skin of resistant bat species, indicate they are commensals. Several of these yeasts may represent novel taxa (Appendix – Supplemental Data) that could be adapted to living on bat skin, and further work is needed to characterize these bat-associated strains. Many yeasts we isolated have been documented from cold regions (e.g. Antarctica and glacial habitats), saline and acidic environments (Middelhoven *et al.*, 1992; Buzzini *et al.*, 2012; Mokhtarnejad *et al.*, 2016), human skin fungal assemblages (Pfaller *et al.*, 2005; Jo *et al.*, 2017; Byrd *et al.*, 2018), and bats in tropical habitats (Grose and Marinkelle, 1966; Grose *et al.*, 1968; Mok *et al.*, 1982; Oyeka, 1994; Gandra *et al.*, 2008; Belisle *et al.*, 2014; Brilhante *et al.*, 2016). These patterns suggest that these yeasts are commensals and thus might affect disease susceptibility.

In this study, we employed a culture-based approach to examine the mycobiome of bats, which has several limitations. Specifically, taxa that are rare, cryptic, fastidious, or unculturable may be overlooked. However, when we compared our culture results to those based on next generation sequencing (NGS) from a subset of samples, we found that our culture-based methods typically detected the fungi most likely to be skin commensals on bats. Further, the use of NGS presents a different set of drawbacks. For example, although NGS can determine relative abundance of various taxa, calculating absolute abundance of particular organisms is more difficult. Samples that do not yield satisfactory sequence data are routinely excluded from further analyses. Meaning that negative datapoints are often discarded rather than given equal weight in analyses. Through the use of zero-inflated models, we were able to retain samples with many

non-detections in our dataset. This demonstrated that WNS-resistant species of bats were significantly more likely to have culturable fungi than WNS-susceptible or WNS-impervious species of bats. Finally, our culture-based analysis yielded isolates of skin commensals for downstream experiments such as *Pd* inhibition assays and further genetic characterization of potentially novel fungal taxa.

Geographic location (site) is an important explanatory variable for the overall diversity and composition of skin fungal assemblages. This pattern was previously found for bat-skin fungal assemblages (Vanderwolf *et al.*, 2015, 2016), bat-skin bacterial assemblages (Avena *et al.*, 2016; Lemieux-Labonté *et al.*, 2016, 2017; Winter *et al.*, 2017), and amphibian skin bacterial assemblages (Loudon *et al.*, 2014; Walke *et al.*, 2014). Microbes that constitute an organism's skin microbial assemblages are primarily drawn from local environments (Loudon *et al.*, 2014), and microbial diversity is influenced by numerous abiotic factors (Fierer and Jackson, 2006). Factors that influence cave fungal assemblages include quantity of organic material and water, cave chemistry, temperature, cave size and depth, and number and diversity of animals (Vanderwolf *et al.*, 2013a). Although we did not characterize environmental fungal assemblages, likely each location we sampled possessed a unique fungal assemblage. Each site was only sampled once and sites within a state were all sampled by the same collection team (which could affect results). Therefore, factors such as day-of-year and month samples were collected, and number of days swabs were stored are proxies for the variable site. Overall, bats in southern states (Alabama, Kentucky, Missouri) had higher fungal abundance than bats in northern states, indicating that temperature or other environmental factors associated with the region could be an important determinant of fungal abundance. However, because many bat species with relatively high fungal abundance (*My. austroriparius*, *My. sodalis*, and *My. grisescens*) were exclusively

sampled in southern states (due primarily to restricted geographic ranges for those bat species), it is difficult to decouple the effects of location and certain environmental parameters from species.

The diversity of fungal species occupying the skin is not the only way in which the microbiome may modulate disease resistance. Instead, resistance may be related to the presence of a single key species that acts as an antagonist to a pathogen. For our second sub-hypothesis, we predicted WNS-resistant species would harbor fungal species that were comparatively rare on WNS-susceptible species, and that these fungi would inhibit *Pd in vitro*. Each WNS-susceptibility group contained at least one fungal species that was enriched within it, all of which were yeasts. The WNS-resistant group had multiple differentially enriched yeasts, one of which, *Cu. moniliiforme*, reduced *Pd* growth *in vitro*, indicating direct antagonistic interactions of this yeast with *Pd* may be a mechanism of WNS-resistance by slowing disease progression. Inhibition was limited to specific pH and salinity conditions, and thus other yeasts might inhibit *Pd* under untested conditions. The production of antifungal compounds by fungi varies with pH, salinity, temperature, and nitrogen source included in media (Marquina *et al.*, 2001; Gasparetti *et al.*, 2006). Studies of physical conditions on the surface of bat skin are necessary to determine the biological relevance of our inhibition assays. Other mechanisms by commensal yeasts may also mediate resistance to *Pd* infection, such as through competition or facilitating immunostimulation in response to infection.

Microbes that inhibit fungi have been proposed for use in WNS control efforts (Hoyt *et al.*, 2015; Cheng *et al.*, 2016), but their effectiveness is difficult to gauge without understanding underlying host factors that influence microbiomes and favor potentially protective microorganisms. We found fungal taxa that were abundant on resistant bats were rare on susceptible bats sampled at the same time from the same locations. This may indicate that

potentially protective microbes are not easily established on WNS-susceptible hosts, which may have different skin chemistries or different microclimate preferences within hibernacula compared to WNS-resistant bats. Therefore, applications of these yeasts on bats may not confer resistance if they cannot colonize, or if environmental conditions are not conducive for yeasts to inhibit *Pd*. Furthermore, although we demonstrated an association between components of skin fungal assemblages and resistance to WNS, it does not prove that commensal fungi directly protect bats from *Pd*. Skin chemistry, physiological, or behavioral traits that promote yeast commensals on some bats may also result in suboptimal conditions for *Pd* growth. Nonetheless, the potential of certain yeasts to protect bats from WNS warrants further investigation, as does the ability to predict WNS-susceptibility of bat populations using skin fungal assemblage characteristics.

TABLES AND FIGURES

Table 2.1**:** Bat species, sample sizes (N), and locations sampled. The number of sites in which each species was sampled per state follows the state abbreviation. The names and coordinates of the collection sites have been withheld due to the sensitive nature of bat hibernacula. AR=Arkansas, WV= West Virginia, IA= Iowa, PA= Pennsylvania, WI= Wisconsin, AL= Alabama, KY= Kentucky, NY= New York, MO= Missouri, OK= Oklahoma.

Bat Species	N	State - # of sites	Years sampled
Corynorhinus rafinesquii	30	AR-2	2017
Co. townsendii virginianus	30	WV-2	2014
Eptesicus fuscus	31	IA-1, PA-1, WI-1, WV-1	2014, 2015, 2016
Myotis austroriparius	32	AL-3	2015
My. grisescens	21	AL-2, KY-1	2014, 2015
My. leibii	4	PA-2	2016
My. lucifugus	43	KY-1, WI-4	2014, 2017
My. lucifugus (WNS-tolerant)	61	NY-2	2014, 2015
My. septentrionalis	39	WI-5	2014, 2015
My. sodalis	28	AL-1, KY-1, MO-1	2014, 2015

Perimyotis subflavus	79	AL-3, KY-1, MO-1, OK-1, WI-3, WV-1	2014, 2015, 2017

Table 2.2**:** Inhibition assays. The mean range in diameter of the zone of inhibition (n=3 replicates for each condition except for the positive control) of *Pseudogymnoascus destructans* (*Pd*) in the presence of indicated yeast strains after 2 weeks of incubation under various conditions in vitro (Sabouraud dextrose medium [SD], brain hear infusion medium [BHI], brain heart infusion agar with 10% sheep blood [BHIB], and yeast morphology medium [YM]). Voriconazole and phosphate-buffered saline with 0.5% Tween 20 (PBST) were used as positive and negative controls, respectively. CI= complete inhibition. [a]Plates checked at 7 weeks due to slow growth of *Pd* on this medium. [b]Inhibition weak with some growth in 'inhibited' area.

Yeast strain	Mean range in diameter of the zone of inhibition (mm)						
	SD	BHI	BHIB	YM, pH 4.5	YM, pH 5.0	YM, pH 5.0, 6% NaCl[a]	YM, pH 7.0
Cutaneotrichosporon moniliiforme (44797-142-2SD)	0	0	0	7	7	0	0
Cu. moniliiforme (44797-153-1DTM)	0	0	0	9.75[b]	7[b]	0	0
Debaryomyces sp. 1 (44797-144-4SD, 44797-166-2SD, 44797-136-2SD)	0	0	0	0	0	0	0
Debaryomyces sp. 3 (44797-66-3SD)	0	0	0	0	0	0	0
D. hansenii (44797-62,2SD, 44797-190-2SD)	0	0	0	0	0	0	0
D. hansenii (type strain NRRL Y-7426)	0	0	0	0	0	0	0
Leucosporidium sp. (44797-83-3SD)	0	0	0	0	0	0	0
Voriconazole	0	16	13	15[b]	20	CI	16
PBST	0	0	0	0	0	0	0

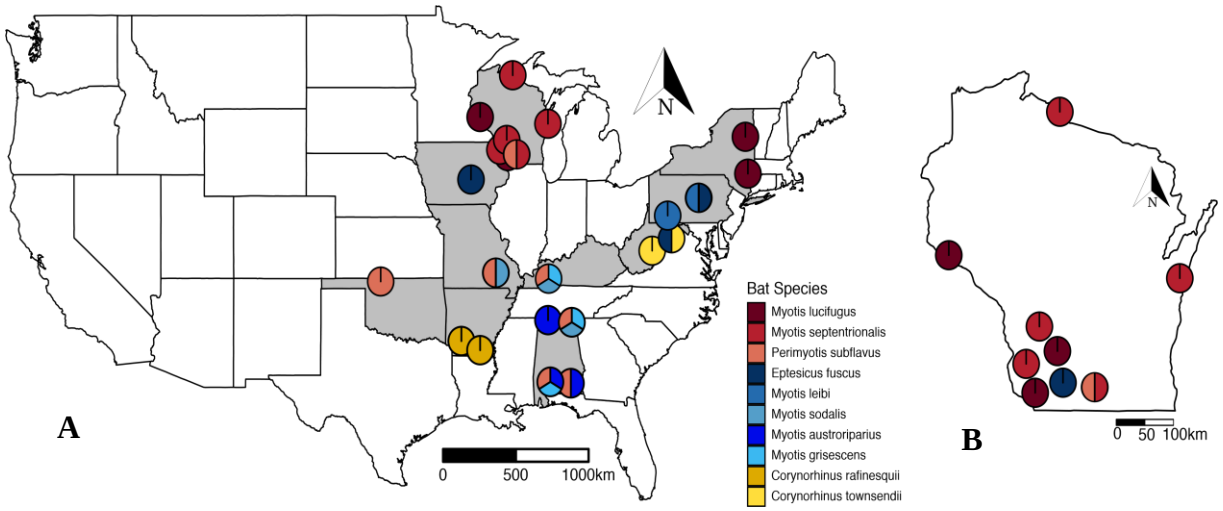

Figure 2.1: Sample site map. A map showing the distribution of sampling sites and the bat species sampled at each site. Each sampling site is represented by a circular chart and the bat species sampled at each site are represented by the colors displayed by its respective chart. Whether or not a species was sampled at a site is displayed as a binary factor, i.e. size of each chart segment is not related to the number of each species sampled at a site. Bat species categorized as white-nose syndrome (WNS)-susceptible are displayed in hues of red, species categorized as WNS-resistant are displayed in hues of blue, and species categorized as WNS-impervious are displayed in hues of yellow. (A) sampling sites and bat species across the United States. Sampled states are shown in grey. (B) A finer scale plot of sampling sites within the state of Wisconsin.

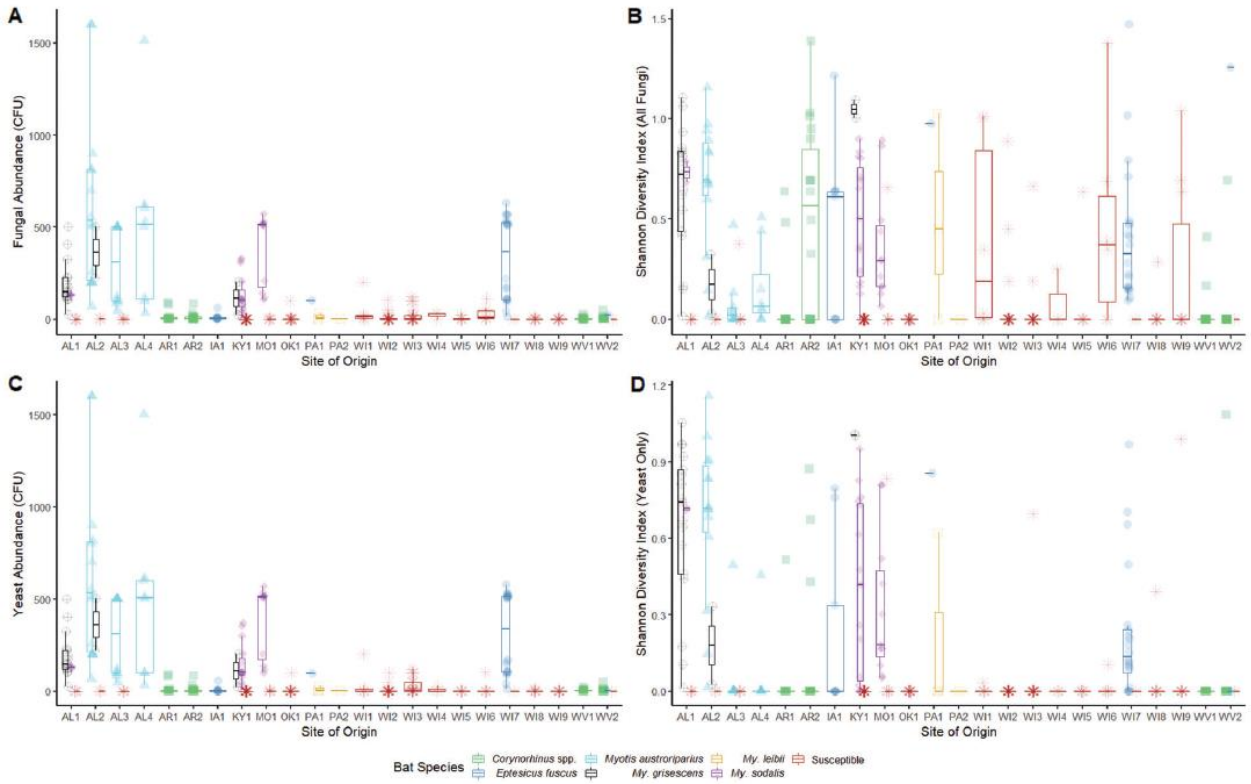

Figure 2.2: Mean counts of fungal abundance and diversity per bat, expressed as colony forming units (CFU), and mean Shannon Index for all fungi (A, B) and yeast only (C, D) on various species of bats at different sites. *Pseudogymnoascus destructans* has been excluded. AR= Arkansas, WV= West Virginia, IA= Iowa, PA= Pennsylvania, WI= Wisconsin, AL= Alabama, KY= Kentucky, NY= New York, MO= Missouri, OK= Oklahoma. *Corynorhinus* spp. = *Co. rafinesquii* and *Co. townsendii virginianus*; WNS-resistant= *Eptesicus fuscus, Myotis austroriparius, My. grisescens, My. leibii, My. sodalis*; WNS-susceptible= *My. lucifugus, My. septentrionalis*, and *Perimyotis subflavus*.

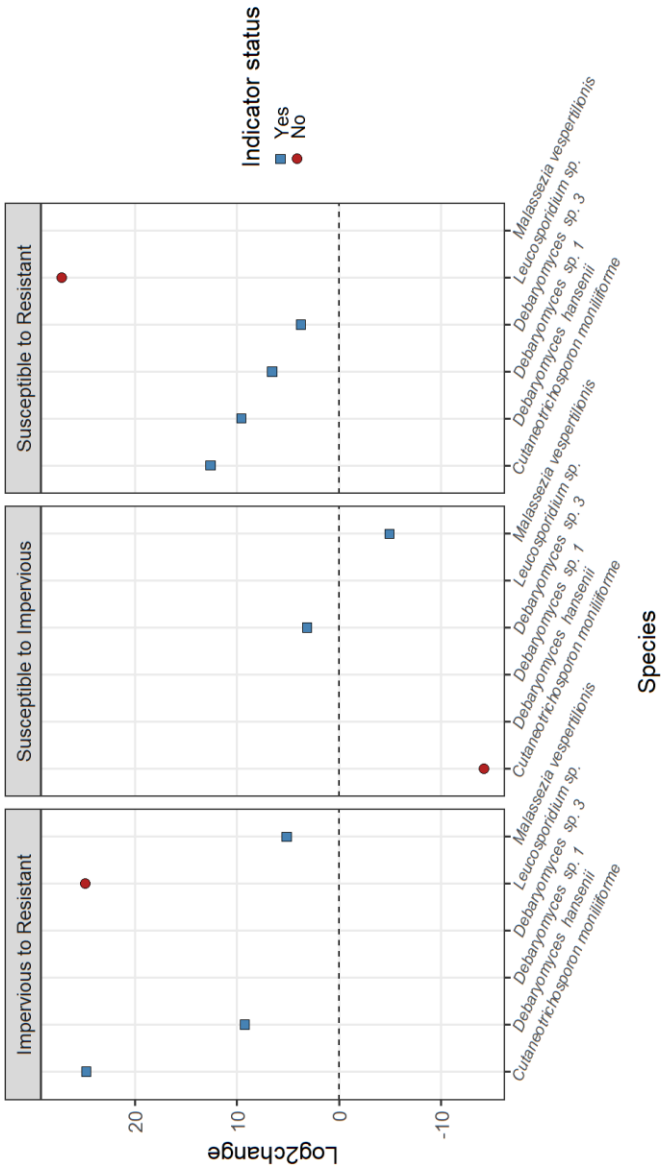

Figure 2.3**:** Operational taxonomic units (OTUs) identified as differentially abundant ($p < 0.05$) among WNS-susceptibility groups in pairwise comparisons. For each pairwise comparison, the WNS-susceptibility group listed first was the baseline and the second was the comparison. Log2change values above zero indicate that the OTUs were more abundant in the comparison group compared to the baseline. OTUs that were significant indicator species (indicator statistic > 0.4, $p < 0.05$) for each WNS-susceptibility group are shown with blue boxes. *Debaryomyces* sp. 3 is an indicator species for both WNS-resistant and WNS-impervious species of bats. All other fungal indicator species are for WNS-resistant species of bats alone. P-values were corrected for multiple hypothesis testing in both tests.

SUPPLEMENTAL MATERIAL CAPTIONS

Table 2.S1: Fungal taxa cultured from the wing surface of bats in the eastern United States. Numbers indicate the number of individual bats from which each fungal operational taxonomic unit (OTU) was cultured. Fungal taxa indicated by * were identified using NCBI BLAST function; all other taxa were identified using UNITE. "NA" indicates the OTU could not be identified with available databases. CORA= *Corynorhinus rafinesquii*, COTO= *C. townsendii virginianus*, EPFU= *Eptesicus fuscus*, MYAU= *Myotis austroriparius*, MYGR= *M. grisescens*, MYLE= *M. leibii*, MYSO= *M. sodalis*, MYLU= *M. lucifugus*, MYLUr= *M. lucifugus* from WNS-resistant colonies in New York, MYSE= *M. septentrionalis*, and PESU= *Perimyotis subflavus*.

Table 2.S2: The top six best zero-inflated models for four different response variables (Shannon Index and abundance, both for all fungi and yeast only) based on Akaike information criterion (AIC). Multiple combinations of variables were tested to determine each variable's relative influence on each response variable. The difference in AIC between the best model (dAIC=0) and next best models are listed. Models with dAIC < 4 are considered identical. Gaussian distributions were the best in all cases. Fungal and yeast abundance are based on counts of colony forming units. Group= white-nose syndrome (WNS)-susceptibility group.

Table 2.S3: Results of the best model for each response variable (Shannon Index and abundance, both for all fungi and yeast only, Table S2). P-values in bold are considered significant (p-value < 0.05).

Table 2.S4: Bat species from which *Pseudogymnoascus destructans* (*Pd*) was cultured. Sites are listed by state followed by the site number. The mean number of colony forming units (CFU) for all fungi totaled over the three agar types used ± the standard deviation is given with the number of *Pd* colonies included in one column and excluded in another. *Pd* was not cultured from *Corynorhinus rafinesquii, C. townsendii, Eptesicus fuscus, M. austroriparius, M. grisescens*, or *M. leibii* during this study. NA = not applicable. MYLU = *M. lucifugus*, MYLUr = *M. lucifugus* from white-nose syndrome (WNS)-resistant colonies New York, MYSE = *M. septentrionalis*, MYSO = *M. sodalis*, PESU = *Perimyotis subflavus*, WI = Wisconsin, KY = Kentucky, NY = New York, AL = Alabama, MO = Missouri, OK = Oklahoma, WV = West Virginia

Table 2.S5: Primers and cycling conditions for phylogenetic analyses.

Table 2.S6: GenBank accession numbers of sequenced loci and models used for phylogenetic analyses.

Table 2.S7: The number of fungal operational taxonomic units (OTUs) per bat comparing culture-independent (CI) to culture-dependent (CD) results. CFU = colony forming units

Table 2.S8: Fungal taxa detected from the wing surface of bats in the Eastern United States using next-generation sequencing. Numbers indicate the number of reads obtained for each fungal operational taxonomic units (OTU). Fungal taxa indicated by * were identified using

NCBI BLAST function; all other taxa were identified using UNITE. "NA" indicates the OTU could not be identified with available databases. Numbers highlighted in green were also detected using culture-dependent methods. MYSO = *Myotis sodalis*, MYSE = *M. septentrionalis*, and MYAU = *M. austroriparius*.

Figure 2.S1: Mean counts, expressed as colony forming units (CFU) for all fungi (A) and yeast only (B) based on white-nose syndrome (WNS)-impervious, WNS-susceptible, and WNS-resistant species of bats. CORA= *Corynorhinus rafinesquii*, COTO= *C. townsendii virginianus*, EPFU= *Eptesicus fuscus*, MYAU= *Myotis austroriparius*, MYGR= *M. grisescens*, MYLE= *M. leibii*, MYSO= *M. sodalis*, MYLU= *M. lucifugus*, MYSE= *M. septentrionalis*, and PESU= *Perimyotis subflavus.*

Figure 2.S2: Images A – C: Yeast cells on the wing surface of an *Eptesicus fuscus* sampled live from a hibernaculum in Wisconsin. Image C also shows smaller bacteria cells. Image D: Hyphae and conidia of *Pseudogymnoascus destructans* on the wing surface of a *Myotis lucifugus* sampled live from a hibernaculum in Wisconsin. Images were taken under high-vac scanning electron microscopy. Scale bar numbers represent the full length of the bar.

Figure 2.S3: Phylogenetic trees from Bayesian analyses of concatenated nucleotide sequences of multiple loci for fungal taxa that were differentially more abundant on bat species that are resistant or impervious to white-nose syndrome. Posterior probabilities are presented at each node if they were greater than 0.95. Type strains for each species of the genus are listed with their associated culture collection identifiers. Potentially novel taxa (as determined by genetic divergence from described species) isolated in this study appear in shaded boxes. (A) Phylogenetic tree for the genus *Debaryomyces* using five loci (internal transcribed spacer [ITS], the D1/D2 region of 26S rDNA [D1D2], second largest subunit of RNA polymerase II [*RPB2*], actin, and mitochondrial cytochrome c oxidase subunit II); (B) phylogenetic tree for the genus *Leucosporidium* using two loci (ITS, D1D2); and (C) phylogenetic tree for the genus *Cutaneotrichosporon* using four loci (ITS, D1D2, *RPB2*, translation elongation factor 1-α).

ACKNOWLEDGEMENTS

We thank the many field biologists who coordinated sampling efforts and collected samples, including Anne Ballmann, Shelly Colatskie, Katie O'Connor, Tony Elliott, Kyle George, Lindsey Heffernan, Carl Herzog, Catherine Hoose, Daryl Howell, Jennifer Redell, Blake Sasse, Michael Scafini, Nick Sharp, Kirk Silas, Craig Stihler, Greg Turner, Paul White, Rebecca Williams, and Michelle Verant. We are most grateful to Rick Lankau for facilitating the culture-independent analysis, Doerte Doepfer and James Paterson for providing statistical advice, and Katie Schmidt and Daniel Taylor for providing technical assistance with laboratory work. We

thank the University of Wisconsin Biotechnology Center DNA Sequencing Facility and Newcomb Imaging Center for providing next-generation sequencing and electron microscopy services, respectively. This work was funded by the U.S. Fish and Wildlife Service, the U.S. Geological Survey, and a Natural Sciences and Engineering Research Council of Canada postgraduate scholarship to KJV. Portions of this work were conducted by KJV and LJC while serving as volunteers at the U.S. Geological Survey. The use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Metadata associated with this publication is available in machine-readable format at https://doi.org/10.5066/P9Y54WW4 [90]. Scripts used in the analysis of this project are available from the GitHub repository URL https://github.com/zoolew/Bat-Wing-Mycobiomes

CHAPTER 3

PREFACE

Title: Mycobiome traits associated with disease tolerance predict many western North American bat species will be susceptible to white-nose syndrome

Authors: Karen J. Vanderwolf, Lewis J. Campbell, Daniel Taylor, Tony L. Goldberg, David S. Blehert, and Jeffrey M. Lorch.

Reference: Published in *Microbiology Spectrum* (2021), **9**(1): e00254-21
https://journals.asm.org/doi/10.1128/Spectrum.00254-21
2020 Impact Factor: 7.171

Contributions: T.L.G., D.S.B, and J.M.L. conceived, directed, and obtained funding for the research presented in this study. D.T. and K.J.V. cultured the fungi and performed PCR. J.M.L. and K.J.V. conceived methods for the inhibition assays. K.J.V. performed the statistical analysis, the inhibition assays, and created Table 3.1, 3.2, 3.3, and Fig. 3.1 and 3.2. L.J.C. provided statistical advice. K.J.V drafted the manuscript and made revisions according to reviewer's recommendations. J.M.L. edited the initial draft. All authors edited the manuscript prior to submission and after subsequent revision according to reviewer's recommendations. C.D. agreed that the contributions of K.J.V. are as stated, above.

CHAPTER 3

Mycobiome traits associated with disease tolerance predict many western North American bat species will be susceptible to white-nose syndrome

Published in *Microbiology Spectrum* (2021), **9**(1): e00254-21

https://journals.asm.org/doi/10.1128/Spectrum.00254-21

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ABSTRACT

White-nose syndrome (WNS), a fungal disease that has caused catastrophic population declines of bats in eastern North America, is rapidly spreading across the continent and now threatens previously unexposed bat species in western North America. The causal agent of WNS, the fungus *Pseudogymnoascus destructans* (*Pd*), can infect many species of hibernating bats, but susceptibility to WNS varies by host species. We previously reported that certain traits of the skin microbiome, particularly yeast diversity and abundance, of bat species in eastern North America are strongly associated with resistance to WNS. Using these traits, we developed models to predict WNS susceptibility of 13 species of western North American bats. Based on models derived from yeast species diversity, only one bat species, *Myotis velifer*, was predicted to be WNS-resistant (i.e., may develop the disease, but with low mortality rates). We also screened yeasts found on western bats for *Pd*-antagonistic properties by spore germination and growth inhibition/competition assays and found the ability of yeasts to inhibit *Pd in vitro* to be strain specific. Similar to results of inhibition assays performed with yeasts isolated from bats in eastern North America, few yeasts isolated from bats in western North America inhibited *Pd in*

vitro. Continued monitoring of western bat populations will serve to validate the accuracy of the mycobiome analysis in predicting WNS-susceptibility, document population and susceptibility trends, and identify additional predictors to assess the vulnerability of naïve bat populations to WNS.

Importance

White-nose syndrome is one of the most devastating wildlife diseases ever documented. Some bat species are resistant to or tolerant of the disease, and we previously reported that certain traits of the skin mycobiome of bat species in eastern North America are strongly associated with resistance to WNS. Predicting which western bat species will be most susceptible to WNS would be of great value for establishing conservation priorities. Based on models derived from yeast species diversity, only one bat species was predicted to be WNS-resistant. High susceptibility to WNS would pose a significant conservation threat to bats in western North America.

INTRODUCTION

White-nose syndrome (WNS) is a devastating disease that has killed millions of hibernating bats in eastern North America (Blehert *et al.*, 2009; U.S. Fish and Wildlife Service, 2012). The causative fungal pathogen, *Pseudogymnoascus destructans* (*Pd*) (Lorch *et al.*, 2011b), was first detected in New York in 2006 and has spread rapidly across North America, threatening some bat species with extinction (Turner *et al.*, 2011; Frick *et al.*, 2015). In 2016, *Pd* was documented in western North America for the first time (Lorch *et al.*, 2016), and several western bat species have subsequently been found with *Pd* on their skin or diagnosed with

clinical WNS (White-nose syndrome response team, 2020). Western North America has higher bat biodiversity than the eastern portion of the continent, and several species are already designated as 'species of concern' by the U.S. Fish and Wildlife Service due to threats such as habitat loss (Adams, 2003; O'Shea *et al.*, 2018). Despite the substantial conservation impact that WNS has had in eastern North America, the nature and severity of WNS in western bat species remains unclear.

Although *Pd* can infect many species of hibernating bats, disease dynamics and population-level impacts of WNS vary by host species (Turner *et al.*, 2011; Frick *et al.*, 2017). Specifically, some bat species in eastern North America are highly susceptible to WNS, others are resistant (develop WNS without experiencing mass mortality), and a few species appear to resist infection altogether (impervious) despite repeated exposure to *Pd* (Turner *et al.*, 2011; Frick *et al.*, 2017). Identifying which western bat species are likely susceptible to WNS prior to the onset of disease would help management agencies set conservation priorities. However, mechanisms of resistance have not been elucidated, making such predictions difficult. Regardless of mechanism, we found in a previous study that certain traits of skin mycobiomes (the fungal component of the microbiome) of eastern bat species were strongly associated with resistance to WNS (Vanderwolf *et al.*, 2021a). WNS-resistant species of bats had higher abundance of fungi, especially yeasts, compared to WNS-susceptible species of bats, and some fungal taxa were enriched on WNS-resistant species of bats (Vanderwolf *et al.*, 2021a). Here we describe skin yeast assemblages on bats in western North America and use data from our previous analyses with eastern bats to construct predictive models to forecast which western bat species may be susceptible or resistant to WNS.

METHODS

Sampling

We collected swab samples from a total of 36 sites in 11 U.S. states and one Canadian province in western North America from 2015 – 2018. In all, we sampled 450 individual bats, representing fourteen species (Table 3.1). We sampled only those western North American bat species known to hibernate (Adams, 2003; O'Shea *et al.*, 2018), because bats that do not hibernate are not known to be susceptible to WNS. Unlike areas of eastern North America where multiple species of bats form large hibernation aggregations (>100 individuals) in caves and mines during winter, hibernation locales for many western bat species are unknown or contain small numbers of bats (O'Shea *et al.*, 2018; Weller *et al.*, 2018). Due to the difficulties of locating many western bat species during the hibernation period, we collected samples year-round, only some of which were at underground sites (caves or mines). Other sampling sites included maternity colonies (bat boxes, bat condos, buildings) and forest landscapes. We conducted sampling under U.S. Geological Survey - National Wildlife Health Center Institutional Animal Care and Use Committee Protocols #EP140212 and #EP081124-A2.

Fungal culture and identification

We collected and processed swabs of bat wings (each individual bat swabbed once) following the methods of Vanderwolf et al. (2021a) with some modifications. First, swabs were stored from two to thirty days (mean 9.5 ± 7.8 SD) before processing due to time variation in shipping samples to the laboratory. Second, we cultured fungi only on Sabouraud dextrose (SD) agar plates with chloramphenicol and gentamicin and Leeming and Notman agar; we did not use dermatophyte test medium because in our previous study this medium contributed little to the

overall assessment of fungal abundance and diversity on bat skin (Vanderwolf *et al.*, 2021a). Finally, we selected only fungi with a yeast morphotype for identification because the abundance of yeasts (colony forming units) was the only component of the mycobiome strongly associated with WNS-susceptibility (Vanderwolf *et al.*, 2021a).

Briefly, we streaked each swab five times, discretely, across the two medium types and incubated plates in darkness at 7 °C (to approximate typical conditions in hibernacula) for 2 months. We checked plates weekly and isolated morphologically unique fungal colonies in pure culture. We counted the number of colonies of each morphotype on each plate weekly for the two-month incubation period, or until confluent growth precluded accurate counts, to determine the number of colony forming units (CFUs). We identified pure cultures by analyzing the full-length internal transcribed spacer (ITS) region of the fungal rRNA gene as previously described (Vanderwolf *et al.*, 2021a). Briefly, after cells were lysed, we performed a polymerase chain reaction targeting ITS using universal fungal primers ITS1-F and ITS4 (cycling conditions: 94 C for 3 min followed by 40 cycles of 94 C for 1 min, 53 C for 1 min, and 72 C for 3 min, with a final extension for 10 min at 72 C) (Lorch *et al.*, 2015). All PCR reactions were conducted using GoTaq® Flexi DNA polymerase (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions, with 0.5-mL template (extracted from fungal isolates using PrepMan™ Ultra reagent (Life Technologies Corporation, Carlsbad, California, USA)) per 25-mL reaction. Sanger sequencing of amplicons was performed at the University of Wisconsin-Madison Biotechnology Center. We deposited sequences in GenBank (accession numbers MK782157 - MK782494) .

Sequences were collapsed into representative operational taxonomic units (OTUs) using USEARCH (Edgar, 2010) with a 97% similarity threshold (O'Brien *et al.*, 2005). We applied a

99% similarity threshold for the genus *Debaromyces* because of minimal genetic variation exhibited in the ITS region among *Debaromyces* species (Martorell *et al.*, 2005). We assigned taxonomy to sequences in R, using the assigntaxonomy function (DADA2 package) (Callahan *et al.*, 2016) with UNITE (Kõljalg *et al.*, 2013; Community, 2017). Some sequences were not identified to genus using UNITE, and we compared these to NCBI's GenBank database using BLAST(Altschul *et al.*, 1990). We manually generated a community matrix of annotated OTUs and their CFUs for each bat.

Inhibition assays

We screened yeasts found on bats for *Pd*-antagonistic properties by spore germination and growth inhibition/competition assays using methods identical to Vanderwolf et al. (2021a). We tested OTUs that were present on ≥ 5 bats each. Briefly, we used different types of media, pH, and salt conditions, including SD (pH 5.6), brain heart infusion (BHI, pH 7.4), BHI with 10% sheep blood (pH 7.4), yeast morphology (YM) medium with the pH adjusted to either 4.5, 5.0, or 7.0 with 0.1 M citrate-phosphate buffer, and YM supplemented with 6% (w/v) NaCl at pH 5.0. We harvested *Pd* conidia, spread 150 µl of *Pd* conidial suspension containing two million conidia onto agar media, placed six pre-sterilized Whatman #1 filter paper discs equidistant from one another on each plate, and inoculated each disc with 8µl suspensions each containing 500,000 cells of different yeast strains. We tested each yeast-strain in triplicate, incubating all plates in the dark at 7 ºC. Negative controls were discs treated with sterile phosphate buffered saline only and positive controls were discs containing voriconazole (30 µg; Sensi-disc; Becton, Dickinson, & co.). We checked plates daily for the first week to assess inhibition of *Pd* germination near the discs, and then weekly for two months or until *Pd* growth

had covered the entire agar surface. We measured zones of inhibition around the discs to the nearest mm after two weeks of incubation.

Statistical analyses

Singletons (fungal OTUs that were isolated from one individual bat) were removed prior to analysis. *Myotis septentrionalis* samples were removed before analysis because we collected samples from only three individuals. We performed all analyses in R (R Core Team, 2020). We calculated the Shannon diversity index of fungi on each bat using the diversity function in the vegan package (Oksanen *et al.*, 2018). To assess which factors influence skin fungal assemblage structure, we constructed a Gaussian zero-inflated model with Shannon Index as the response variable and bat species (13 level factor), state (12 level factor), the number of days swabs were stored, whether samples were collected at underground sites (caves or mines, binary factor), and season (4 level factor) as explanatory variables (package glmmTMB) (Brooks *et al.*, 2017). We defined 'winter' as December – February, 'spring' as April – May, 'summer' as June – August, and 'fall' as September – October. No samples were collected in March or November. A separate model with yeast CFUs (abundance) as the response variable and identical explanatory variables was also constructed. We used the function AICtab (package bbmle) (Bolker and Team, 2017) to compare model Akaike information criteria (AIC) values. We determined the best data transformation with the transformTukey function (package rcompanion) (Mangiafico, 2019) for each response variable. Lambda was 0.4 and 0.2 for Shannon Diversity and yeast abundance, respectively. We tested multicollinearity among the variables using the 'corvif' function (Zuur *et al.*, 2009).

To determine if wing fungal assemblage composition varied among WNS-susceptibility groups, bat species, and sites, we implemented a non-parametric permutational multivariate analysis of variance (PERMANOVA) on abundance based (CFU) Bray-Curtis dissimilarity coefficients using the function ADONIS (Vegan). As Bray-Curtis dissimilarity values cannot be calculated for samples that have no composition, we first removed individual bats with no cultured fungi and bats from which the only fungus cultured was the single representative of that OTU within our dataset (n=17 *Antrozous pallidus*, n=57 *Corynorhinus townsendii*, n=1 *E. fuscus*, n=6 *Euderma maculatum*, n=14 *Myotis californicus*, n=16 *My. ciliolabrum*, n=33 *My. evotis*, n=11 *My. lucifugus*, n=10 *My. thysanodes*, n=34 *My. volans*, n=22 *My. yumanensis*, and n=12 *Parastrellus hesperus*). We ran PERMANOVA for 1,000 iterations, and report R^2 values when the variable enters the model last.

To predict WNS-susceptibility in western bat species, we constructed a logistic regression model. We built our model using previously published data on the abundance of yeasts (colony forming units per bat) within skin mycobiomes of bat species of known WNS-susceptibility sampled in eastern North America (Vanderwolf *et al.*, 2021a). We filtered the dataset as previously described (Vanderwolf *et al.*, 2021a), and excluded the yeast *Malassezia vespertilionis* because it was commonly found on both WNS-susceptible and WNS-resistant bat species in the eastern U.S. (Vanderwolf *et al.*, 2021a), and thus is not a good predictor of WNS-susceptibility status. We removed WNS-impervious bat species (*Corynorhinus townsendii* and *Co. rafinesquii*) from the eastern North American dataset prior to constructing the model because WNS-susceptible and WNS-impervious bat species had similar yeast abundance on their skin (Vanderwolf *et al.*, 2021a) and were therefore uninformative for model purposes. We fitted WNS susceptibility status (binomial: resistant or susceptible) as our response variable and yeast

abundance as the sole predictor variable. We assessed model performance with 5-fold cross validation using the function 'cross_validate' in the cvms package (Olsen and Zachariae, 2020). We report the Matthews correlation coefficient because it is considered the best metric for establishing the quality of a binary classifier (Chicco and Jurman, 2020). The eastern dataset had unequal numbers of the two susceptibility groups, but the Matthews correlation coefficient can be used even if classes are of very different sizes (Boughorbel *et al.*, 2017). A coefficient of +1 represents perfect prediction, 0 no better than random prediction, and −1 indicates total disagreement between prediction and observation. We used the 'predict' function of the stats package (R Core Team, 2020) to predict the susceptibility of individuals of unknown susceptibility in the western North America dataset. If >50% of individuals of a given western bat species were predicted as WNS-resistant, we considered the species WNS-resistant, otherwise the species was considered WNS-susceptible. In addition to yeast abundance, we constructed additional models that used the presence or absence of four yeast taxa that were enriched on WNS-resistant species of bats in eastern North America as binary predictor variables of WNS-susceptibility for western bat species in separate binomial models constructed as described above. The four yeast taxa were *Cutaneotrichosporon moniliiforme*, *Debaryomyces* sp. 1, *Debaryomyces* sp. 3, and *Debaryomyces hansenii*.

RESULTS

We isolated a total of 98 yeast OTUs from 52 genera (Table 3.S1). We found that 57.7% of yeast OTUs were cultured from a single individual bat and 86.6% of yeast OTUs were cultured from ≤ five individual bats each (Table 3.S1).The most common yeast genera were *Debaryomyces* spp. (isolated from 27.7% of the 447 bats sampled), *Aureobasidium* spp. (17.2%),

Vishniacozyma spp. (6.5%), *Filobasidium* spp. (3.4%), and *Holtermanniella* sp. (2.9%). Bats had a mean of 0.9 ± 1.1 (standard deviation) yeast OTUs and 41.4 ± 97.5 yeast CFUs per individual not including singleton OTUs.

The Shannon Diversity Index and number of CFUs (abundance) of yeast assemblages on bat wings varied among species, seasons, sampling location, and whether bats were sampled at underground sites (Figure 3.1, Table 3.S2). Shannon Diversity decreased as the number of days samples were stored before processing increased, although this had no effect on yeast abundance (Table 3.S2). Both Shannon Diversity and yeast abundance were higher at underground sites compared to samples collected at other areas (e.g. forest sites, maternity roosts). Samples were collected at underground sites in all seasons, but winter samples were exclusively collected at underground sites. Shannon Diversity and yeast abundance were highest in winter and lowest in summer. *Myotis velifer* and *E. fuscus* had the highest Shannon Diversity and yeast abundance compared to the other tested bat species (Figure 3.1, Table 3.S2). *Corynorhinus townsendii* and *My. yumanensis* were more likely to have no yeast than the other tested bat species.

Skin fungal assemblage composition was significantly associated with bat species (pseudo-$F_{12,184}$=1.79, R^2=0.078, p=0.001), collection location (pseudo-$F_{11,184}$=2.58, R^2=0.103, p=0.001), season of collection (pseudo-$F_{3,184}$=2.35, R^2=0.026, p=0.001), and whether samples were collected at an underground site (pseudo-$F_{1,184}$=1.84, R^2=0.007, p=0.008), but not with the number of days swabs were stored before processing (pseudo-$F_{1,184}$=1.25, R^2=0.005, p=0.187). Several isolates of *Aureobasidium* spp. showed weak inhibition of *Pd* growth under a variety of conditions (Table 3.3). Results varied depending on the medium type used, including results with the positive control voriconazole. *Holtermanniella takashimae* only inhibited *Pd* growth on Sabouraud dextrose medium (Table 3.3). Most of the tested yeast strains showed no inhibition of

Pd. As demonstrated with *Aureobasidium* spp., the ability of yeast to inhibit *Pd* growth *in vitro* is strain specific. Although strains of *Cutaneotrichosporon moniliiforme* isolated from eastern bats inhibited *Pd in vitro* (Vanderwolf *et al.*, 2021a), strains of *Cu. moniliiforme* isolated from western bats showed no inhibition.

The four yeast taxa that were enriched on WNS-resistant species of bats in eastern North America were not as abundant on western bats: *Cu. moniliiforme* was isolated from 17.3% of eastern bats and 2% of western bats, *Debaryomyces* sp. 1 (14.3% east and 6.3% west), *Debaryomyces* sp. 3 (12.3% east and 8.7% west), and *Debaryomyces hansenii* (29.1% east and 12.5% west). Based on models of the presence of three of the four yeast taxa enriched on resistant bat species in eastern North America, the only western bat species predicted to be WNS-resistant was *Myotis velifer* (Figure 3.2, Table 3.2). The remaining 12 species sampled were predicted to be WNS-susceptible based on all criteria examined, although some species (e.g., *E. fuscus*) approached the percent cut-off for WNS-resistance in some models (Figure 3.2).

DISCUSSION

Based on models of the presence of three yeast taxa enriched on resistant bat species in eastern North America, the only western bat species predicted to be WNS-resistant was *Myotis velifer* (Figure 3.2, Table 3.2). Unlike the other species, *My. velifer* samples all originated from the same site, which may bias results. Furthermore, the species was considered WNS-susceptible based upon the overall yeast abundance model, making it difficult to predict WNS-associated impacts on *My. velifer* populations based on mycobiome traits. The remaining 12 species sampled were predicted to be WNS-susceptible based on all criteria examined, although some

species (e.g., *E. fuscus*) approached the percent cut-off for WNS-resistance in some models (Figure 3.2).

Aside from *E. fuscus*, predictions of susceptibility for western populations of species with continental distributions (e.g., *My. lucifugus*) were consistent with the known susceptibilities of their eastern counterparts. However, it is important to note that the mycobiome characteristics we used in our predictions do not distinguish WNS-susceptible from WNS-impervious bat species in eastern North America, as both have low yeast diversity and abundance on their skin. This means that a bat classified as 'susceptible' by our model may be either susceptible or impervious. Western populations of *Co. townsendii* were classified as 'susceptible' by our model, but they are likely to be WNS-impervious based on observations from eastern North America (Bernard *et al.*, 2015). Haase et al. (2020) predicted that *My. velifer, E. fuscus*, and *Co. townsendii* would have a higher probability of survival when infected with *Pd* compared to other bat species in the western USA, such as *My. lucifugus* and *My. evotis*, based on models with body mass and hibernacula microclimate. Aside from bat species that do not hibernate for long periods, currently the only known WNS-impervious bat species are in the genus *Corynorhinus*. *Corynorhinus* is phylogenetically divergent from *Myotis*, among which there are no known impervious taxa. Thus, our predictions may be more accurate for species of *Myotis* and *Parastrellus* (the latter of which is a sister genus to *Perimyotis* - an eastern North American taxon that is highly susceptible to WNS (Adams, 2003; Turner *et al.*, 2011)). Several western species included in our model do not fall into the WNS-impervious category because individuals of these species have been confirmed to develop WNS. For example, *My. velifer, My. evotis, My. thysanodes, My. volans* and *My. yumanensis* have recently been confirmed to develop WNS, although population declines have not yet been observed (White-nose syndrome response team,

2020), indicating these species are not WNS-impervious. There is, however, more uncertainty in predictions for some other western bat species for which no close relatives occur in eastern North America (e.g., *Euderma maculatum*, *Antrozous pallidus*), and these species may be WNS-impervious independent of their mycobiomes.

If our model predictions are accurate, more western bat species in North America could be WNS-susceptible than eastern bat species. However, there are several caveats. First, the mycobiome is but one of several factors that influences host susceptibility to WNS. Our models intentionally focused on the mycobiome because of its association with host susceptibility in our previous study (Vanderwolf *et al.*, 2021a), but we fully acknowledge the multifactorial nature of host susceptibility to fungal pathogens. Second, the traits used in our predictive models likely vary temporally and spatially beyond the parameter ranges that we measured and modeled. For instance, the bacterial microbiome of several western bat species differed between individuals sampled in underground (i.e., caves and mines) versus surface sites (Winter *et al.*, 2017). Many bats sampled for our project were captured from the surface and during the non-hibernation season (i.e., when the species would not be at risk of developing WNS) due to the difficulty of locating hibernacula for most western bat species, and we found that Shannon Diversity and yeast abundance and composition differed between bats sampled at underground sites compared to surface sites.

Yeast assemblages on bat skin are believed to represent primarily commensal rather than transient inhabitants compared to filamentous fungi (Vanderwolf *et al.*, 2021a), and thus are more likely to be permanent residents of the skin. Although multiple yeast taxa were shared between bats in both eastern and western North America, several differed in abundance or were unique to the west. Of the five yeast taxa associated with WNS-resistant eastern bat species

(Vanderwolf *et al.*, 2021a), one taxon was detected on a single individual bat, and the other four taxa were generally uncommon on western bats. Whether yeast taxa unique to the west are associated with WNS-resistance is unknown, although our inhibition assays uncovered few yeasts that inhibit *Pd* growth *in vitro*. Potentially other microbes on the skin, such as bacteria, perform this role (Hoyt *et al.*, 2015).

Several yeast strains isolated from bats in western North America inhibited *Pd* growth *in vitro. Aureobasidium* spp. were more common on bats in western North America than in the east, and several isolates of *Aureobasidium* showed weak inhibition of *Pd* growth under a variety of conditions (Table 3.3). *Holtermanniella takashimae* only inhibited *Pd* growth on Sabouraud dextrose medium (Table 3.3). As demonstrated with *Aureobasidium* spp., the ability of yeast to inhibit *Pd* growth *in vitro* is strain specific. *Cutaneotrichosporon moniliiforme* isolated from bats in the east inhibited *Pd* growth on two media types (Vanderwolf *et al.*, 2021a), but *Cu. moniliiforme* isolated from bats in western North America did not inhibit *Pd* growth on any of the same tested media types (Table 3.3). Similar to results of inhibition assays performed with yeasts isolated from bats in eastern North America, few yeasts isolated from bats in western North America inhibited *Pd* growth *in vitro*. It is unknown which yeasts could inhibit *Pd* on bat wings given that *in vivo* conditions would differ from *in vitro* conditions and the chemical environment on bat wings is largely unknown. Finally, it is plausible that yeast assemblage characteristics common to resistant bat species do not directly modulate disease susceptibility but are rather a result of other behavioral or physiological processes that also influence resistance (Vanderwolf *et al.*, 2021a).

Yeast assemblage traits used in the predictive models could also exhibit variation due to geography and associated host genetics. Eastern and western populations of North American bat

species with continental-wide distributions are genetically distinct, often exhibiting higher genetic diversity in the west (Neubaum *et al.*, 2007; Vonhof *et al.*, 2016). As such, susceptibility to WNS could be different between eastern and western populations and even within western populations. There was greater intraspecific variation in yeast community traits observed in some western bat species compared to eastern species. For example, 7.7% of individuals from western populations of *My. lucifugus*, a WNS-susceptible species, were classified as resistant by the yeast abundance model whereas 0% of individuals from eastern populations had yeast abundance traits consistent with WNS- resistance. The disparity in yeast abundance profiles between susceptible and resistant species of bats in eastern North America creates uncertainty between predicted and observed disease susceptibilities.

Wildlife diseases such as WNS pose significant management challenges due to the difficulty of treating free-ranging populations, especially once a disease has become established. Thus, management strategies focusing on prevention or early intervention hold the most promise for controlling wildlife diseases before they impact new populations or emerge in new locations. Such proactive strategies, however, require intimate knowledge of a hosts' susceptibility to a pathogen. The microbiome is increasingly recognized as an important moderator of disease (Kearns *et al.*, 2017; Kong and Morris, 2017) but has rarely been used to predict host susceptibility. We have attempted to forecast disease impacts on novel host species using traits of the mycobiome. We emphasize that our predictions will likely change as new data on bat skin microbiomes are generated, such as the role of bacteria and viruses. More data will help assess the utility of skin microbiomes in forecasting disease susceptibility. Despite uncertainties, our results provide information that may assist wildlife managers and strategic decision-makers. Specifically, our model predicts that WNS will likely pose a continent-wide conservation threat

to *Myotis* spp. and the disease may have more severe ecological impacts in western North America due the greater number of potentially susceptible host species. Continued monitoring of western bat populations will serve to document population and susceptibility trends, determine the accuracy of the mycobiome in predicting WNS- resistance, and identify additional predictors to assess the vulnerability of naïve bat populations to WNS.

TABLES AND FIGURES

Table 3.1**:** Bat species, sample sizes (N), and locations sampled in western North America. The names and coordinates of the collection sites have been withheld due to the sensitive nature of bat hibernacula. 'East' refers to eastern North America. AZ=Arizona, CA= California, CO= Colorado, BC= British Columbia, ID= Idaho, MT= Montana, NV= Nevada, OR=Oregon, TX= Texas, UT= Utah, WA= Washington, and WY= Wyoming. ?= unknown susceptibility to white-nose syndrome (WNS). *= Species in which WNS has been confirmed, indicating the species is either susceptible or resistant (but not impervious) to the disease.

Bat Species	WNS-susceptibility	N	State/Province- # of sites
Antrozous pallidus	?	39	AZ-1, CA-1, OR-1
Corynorhinus townsendii	impervious in East	83	CA-2, CO-1, ID-1, MT-1, NV-1, UT-1, WY-2
Eptesicus fuscus	resistant in East	9	AZ-1, MT-1, WA-1
Euderma maculatum	?	8	BC-1, NV-1
Myotis californicus	?	34	AZ-1, CA-3, OR-2
M. ciliolabrum	?	36	AZ-1, BC-1, CA-2, ID-1, NV-2, UT-1, WY-2
M. evotis	?*	47	BC-2, CA-2, ID-1, MT-1, NV-5, OR-2
M. lucifugus	susceptible in East	13	BC-2, ID-1
M. septentrionalis	susceptible in East	3	BC-1
M. thysanodes	?*	32	AZ-1, CA-3, OR-1, TX-1
M. velifer	?*	31	TX-1
M. volans	?*	43	AZ-1, BC-2, CA-1, MT-1, NV-4, OR-3
M. yumanensis	?*	55	BC-1, CA-4, WA-2
Parastrellus hesperus	?	17	AZ-1, NV-1

Table 3.2**:** Results of our model predictions of which bat species in western North America will be resistant to white-nose syndrome (WNS) based on different mycobiome characteristics, including yeast abundance cultured from skin swabs (counts of colony forming units) and the presence of four yeast taxa that were differentially abundant on WNS- resistant bat species in eastern North America (as determined by Vanderwolf et al. (K. J. Vanderwolf *et al.*, 2021a)). A Matthews correlation coefficient of +1 represents perfect prediction, 0 no better than random prediction, and −1 indicates total disagreement between prediction and observation for each model. 'N' indicates sample size.

Bat Species	**N**	**Yeast abundance**	***Cutaneotrichosporon moniliiforme***	***Debaryomyces* sp. 3**	***Debaryomyces* sp. 1**	***Debaryomyces hansenii***
Matthews Correlation Coefficient		0.80	0.48	0.05	0.31	0.85
Antrozous pallidus	39	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
Corynorhinus townsendii	83	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
Eptesicus fuscus	9	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
Euderma maculatum	8	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
Myotis californicus	34	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
M. ciliolabrum	36	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
M. evotis	47	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
M. lucifugus	13	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
M. thysanodes	32	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
M. velifer	31	**Susceptible**	**Susceptible**	**Resistant**	**Resistant**	**Resistant**
M. volans	43	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**
M. yumanensis	55	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**

Parastrellus hesperus	17	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**	**Susceptible**

Table 3.3**:** The mean diameter of the zone of inhibition (n=3 replicates for each condition except positive controls) of *Pseudogymnoascus destructans* (*Pd*) in the presence of indicated yeast strains after 2 weeks incubation at 7ºC under various conditions in vitro (Sabouraud dextrose medium [SD], brain heart infusion medium [BHI], brain heart infusion agar with 10% sheep blood [BHIB], and yeast morphology medium [YM]). Voriconazole and phosphate-buffered saline with 0.5% Tween 20 (PBST) were used as positive and negative controls, respectively. *Filobasidium magnum* (46370-1167-0LNA), *Filobasidium* sp. (46379-1438-2LNA), *Vishniacozyma* sp. cluster9 (46388-1843-1LNA), *Blastobotrys buckinghamii* (45699-84-1LNA), *Vishniacozyma victoriae* cluster11 (45701-666-2SD), *Debaryomyces hansenii* (45701-670-3SD, 45701-677-1SD), *Debaryomyces* sp. 1 (45702-288-1SD), *Debaryomyces* sp. 3 (45698-832-4SD), *Trichosporon otae* (44797-05-2SD), and *Cutaneotrichosporon moniliiforme* (44797-11-1LNA) showed no inhibition under any conditions and thus do not appear in the table.

Yeast strain	Mean diameter of the zone of inhibition (mm)						
	SD	BHI	BHIB	YM, pH 4.5	YM, pH 5.0	YM, pH 5.0, 6% NaCl[a]	YM, pH 7.0
Aureobasidium pullulans 46370-1185-2SD	2[b]	0	0	3[b]	0	0	0
Aureobasidium sp. 46370-1064-1LNA	2[b]	0	0	0	0	0	0
Aureobasidium pullulans 45699-972-4aSD	2, 12[b]	12[b]	7[b]	2, 16[b]	7[b]	0	3[b]
Aureobasidium pullulans 46379-835-2LNA	0	0	0	0	0	5[b]	0
Holtermanniella takashimae 45701-673-1SD	4[b]	0	0	0	0	0	0
Voriconazole	6[b]	25	12[b]	10	19	CI	17
PBST	0	0	0	0	0	0	0

[a]plates checked at 7 weeks due to slow growth of *Pd* on this medium
[b]inhibition weak with some growth in 'inhibited' area
CI=complete inhibition

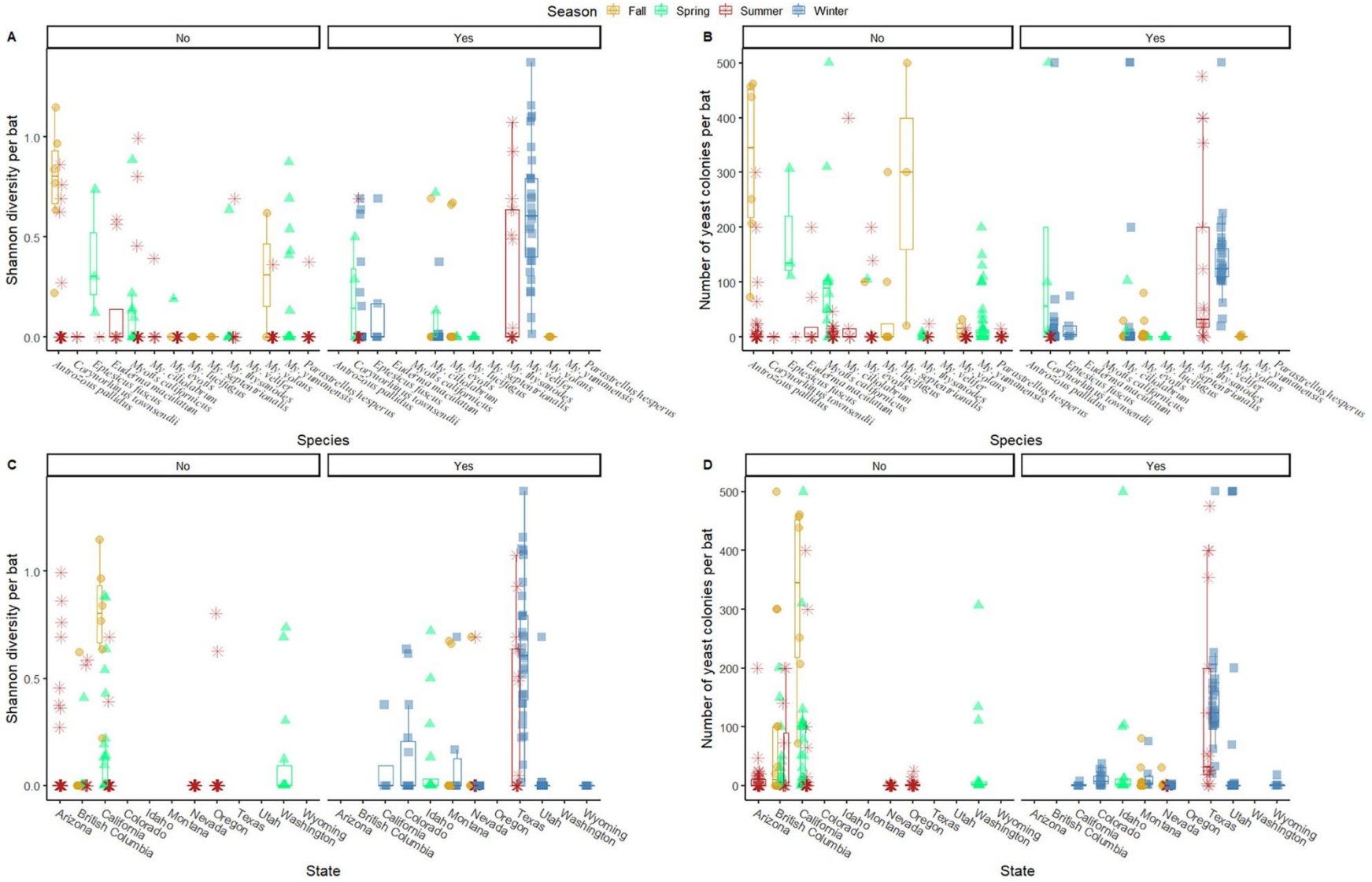
Season
Fall
Spring
Summer
Winter
A
B
C
D
No
Yes
Shannon diversity per bat
Number of yeast colonies per bat
Species
State
Antrozous pallidus
Corynorhinus townsendii
Eptesicus fuscus
Euderma maculatum
Myotis californicus
Myotis ciliolabrum
Myotis evotis
Myotis lucifugus
Myotis septentrionalis
Myotis thysanodes
Myotis velifer
Myotis volans
Myotis yumanensis
Parastrellus hesperus
Arizona
British Columbia
California
Colorado
Idaho
Montana
Nevada
Oregon
Texas
Utah
Washington
Wyoming

Figure 3.1**:** The Shannon Diversity Index (panels A, C) and number of yeast colony forming units (B, D) for each individual bat by species (A, B) and location of sample collection (C, D) colored by season of collection is shown for western North America. Whether a sample was collected at an underground site (cave or mine) is indicated by 'yes' and 'no'. We defined 'winter' as December – February, 'spring' as April – May, 'summer' as June – August, and 'fall' as September – October.

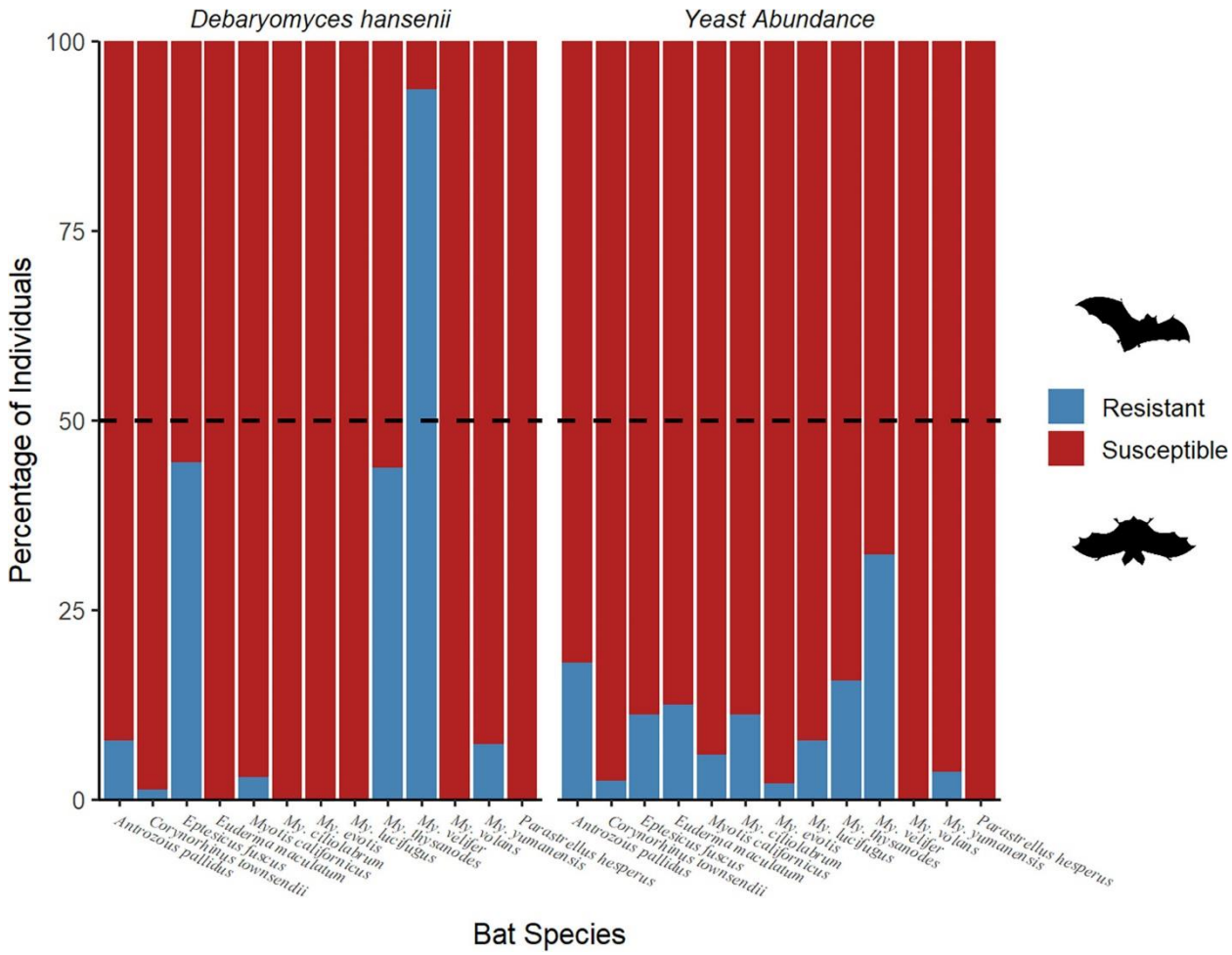

Figure 3.2: The percentage of individuals of each bat species sampled in western North America predicted to be resistant or susceptible to white-nose syndrome based on the presence of *Debaryomyces hansenii* and overall yeast abundance (counts of colony forming units per bat) cultured from wing swabs. Species with >50% of individuals predicted as resistant (blue bar above 50) were considered resistant.

SUPPLEMENTARY MATERIAL CAPTIONS

Table 3.S1: Yeast taxa cultured from the wing surface of bats in western North America. Aside from total sample size for each species (row 2), numbers indicate the number of individual bats

each fungal operational taxonomic unit (OTU) was cultured from. In most cases multiple OTUs were cultured from an individual bat (thus, sums may exceed the sample size listed in row 2). Fungal taxa indicated by * were identified using NCBI BLAST function, all other taxa were identified using UNITE. "NA" indicates the OTU could not be identified with available databases.

Table 3.S2: Results of Gaussian zero-inflated models with two response variables: Shannon Diversity Index and number of yeast colony forming units representing yeast abundance. 'Days' refers to how many days samples were stored before processing. 'Cave' represents whether samples were collected at underground sites (caves or mines). 'Location' refers to the state or province of sample collection in western North America. We defined 'seasons' as winter (December – February), spring (April – May), summer (June – August), and fall (September – October). Significant p-values (<0.05) are in bold. The zero-inflation model for the Shannon Diversity model (~1) estimate = -26.64, standard error = 44616.95, p-value= 1.

DATA AVAILABILITY STATEMENT

Metadata associated with this project is available at

https://www.sciencebase.gov/catalog/item/5cf6c524e4b0d63728b9b463.

Fungal ITS sequences are available in NCBI GenBank, accessions MK782157 - MK782494.

ACKNOWLEDGEMENTS

We thank the many field biologists that collected samples and coordinated sample collection, including B. Abel, Chris Anderson, Linda Anger, Anne Ballmann, Laura Beard, B. Bybee, Allen Calvert, Keith Day, Krysta Demere, R Doening, Julia Ersan, Jennifer Fox, Nate Fuller, Kyle George, Katie Gillies, Mary Gilmore, Catherine Haase, Anthony Kerwin, Cori Lausen and Wildlife Conservation Society Canada, Liam McGuire, Angie McIntire, Eathan McIntyre, Sarah Olson, Gabriel Antonio Reyes, David Riggs, Kirk Silas, Shawn Thomas, Abby Tobin, Ted Weller and the Redwood Hoary Bat Crew, Jason Williams, Rebecca Williams, and R. Winton. David Blehert is a fellow of the CIFAR Program, Fungal Kingdom: Threats and Opportunities. Bat images used in Fig 3.1 were free-for-use through a Creative Commons license (Attribution-

NonCommercial 4.0 License, http://getdrawings.com/bat-silhouette-images; Natasha Sinegina, Creative Commons Attribution-Share Alike 4.0 License; http://www.supercoloring.com/silhouettes/bat). Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Funding: This work was funded by the U.S. Fish and Wildlife Service, the U.S. Geological Survey, and a Natural Sciences and Engineering Research Council of Canada postgraduate scholarship to KJV. Some samples used in this study were collected as part of a project funded by the Department of Defense Strategic Environmental Research and Development Program, under Contract Number W912HQ-16-C-001. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

CHAPTER 4

PREFACE

Title: Skin pH varies among bat species, seasons, and between wild and captive bats

Authors: Karen J. Vanderwolf, Christopher J. Kyle, Paul A. Faure, Donald F. McAlpine, and Christina M. Davy.

Reference: Published in *Conservation Physiology* (2021), **9**(1): coab088
https://academic.oup.com/conphys/article/9/1/coab088/6454461?login=true
2020 Impact Factor: 3.097

Contributions: C.M.D., K.J.V., D.F.M., and C.J.K. conceived and obtained funding for the research presented in this study. K.J.V. took measurements of bat wings (with help from numerous people listed in the acknowledgements), performed statistical analysis, and created Table 4.1, 4.2, 4.S1, 4.S2, and Fig. 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.S1, 4.S2, 4.S3, and 4.S4. Erica Newton helped create Fig 4.2. K.J.V drafted the manuscript and made revisions according to reviewer's recommendations. C.M.D. edited the initial draft. All authors edited the manuscript prior to submission and after subsequent revision according to reviewer's recommendations. C.M.D. agreed that the contributions of K.J.V. are as stated, above.

CHAPTER 4

Skin pH varies among bat species, seasons, and between wild and captive bats

Published in *Conservation Physiology* (2021), **34**(1): 1-12

https://academic.oup.com/conphys/article/9/1/coab088/6454461?login=true

https://doi.org/10.1093/conphys/coab088

ABSTRACT

Skin is a key aspect of the immune system in the defense against pathogens. Skin pH regulates the activity of enzymes produced both by hosts and by microbes on host skin, thus implicating pH in disease susceptibility. Skin pH varies inter- and intra- specifically and is influenced by a variety of intrinsic and extrinsic variables. Increased skin alkalinity is associated with a predisposition to cutaneous infections in humans and dogs, and interspecific and inter-individual variation in skin pH is implicated in differential susceptibility to some skin diseases. The cutaneous pH of bats has not been characterized, but is postulated to play a role in susceptibility to white-nose syndrome (WNS), a fungal infection that has decimated several Nearctic bat species. We used non-invasive probes to measure pH of bat flight membranes in five species with differing susceptibility to WNS. Skin pH ranged from 4.67 to 8.59 and varied among bat species, geographic locations, body parts, age-classes, sexes, and seasons. Wild *Eptesicus fuscus* were consistently more acidic than wild *Myotis lucifugus, M. leibii,* and *Perimyotis subflavus*. Juvenile bats had more acidic skin than adults during maternity season but did not differ during swarming. Male *M. lucifugus* were more acidic than females during maternity season, yet this trend reversed during swarming. Bat skin was more acidic in summer compared to winter, a pattern also reported in humans. Skin pH was more acidic in captive

versus wild *E. fuscus*, suggesting environmental impacts on skin pH. The pH of roosting substrates affects skin pH in captive bats and may partially explain seasonal patterns in wild bats that use different roost types across seasons. Future research on the influence of pH on microbial pathogenic factors and skin barrier function may provide valuable insights on new therapeutic targets for treating bat skin conditions.

INTRODUCTION

Skin is a complex physical barrier and chemical landscape representing one of the first lines of defense hosts have against pathogens (Elias, 2005; Byrd *et al.*, 2018). Despite direct environmental exposure to microbiota, skin is largely unsuitable for microbial colonization, unlike mucosal surfaces (Chen *et al.* 2018). Physiological properties of the skin can affect innate immune function in addition to influencing the growth of microbes (Diamond *et al.*, 2009; Jantsch *et al.*, 2015). Skin surface defenses against microbial invasion include the combined effects of desiccation, epidermal desquamation, acidic pH, nutrient limitations, commensal microbes, antimicrobial lipids (sebum), and antimicrobial peptides (Harder *et al.* 2013; Naik *et al.* 2012). Disruption of these defenses can affect susceptibility to cutaneous diseases (Harder *et al.* 2013; Naik *et al*. 2012).

Cutaneous pH may alter pathogen virulence or host susceptibility because pH affects enzyme production, activation, and efficiency in hosts as well as their commensal microbes and pathogens (Elias, 2005). The pH of skin influences at least four key epidermal functions: permeability barrier homeostasis, integrity/cohesion (desquamation), initiation of inflammation, and antimicrobial defense (Hachem *et al.*, 2003; Elias, 2005). Recovery of human and laboratory mice skin barrier function after injury proceeds normally at an acidic pH (< 6 pH), but is delayed

at a neutral pH (*i.e.* 7 – 7.4 pH) as a result of impaired post-secretory processing of extracellular lipids in the lower stratum corneum by pH-dependent enzymes (Behne *et al.*, 2002; Proksch and Neumann, 2019). Alkaline (basic) skin pH can increase virulence of several fungal pathogens by facilitating penetration into host surfaces and evasion of immune responses (Vylkova, 2017). Attempts to induce *Candida albicans* (pathogenic yeast) lesions were more successful on human skin alkalized to 6.0 pH with topical products compared to unaltered skin at 4.5 pH (Runeman *et al.*, 2000). This pattern was not caused by inhibited growth of *C. albicans*, but instead was though to be due to pH dependence of either the yeast's virulence capacity or modulations of the host's defenses (Runeman *et al.*, 2000). Increased skin alkalinity in humans, laboratory mice, and dogs is associated with a predisposition to cutaneous infections such as bacterial pyoderma, multiple types of dermatitis, acne, eczema, candidiasis, tinea, and diaper rash (Chikakane and Takahashi, 1995; Matousek and Campbell, 2002; Matousek *et al.*, 2003; Hatano *et al.*, 2009). These findings suggest skin pH may also be important in cutaneous wildlife diseases such as amphibian chytridiomycosis and bat white-nose syndrome (WNS), both of which have devastated some species but not others (Fisher *et al.*, 2016). Indeed, *Batrachochytrium dendrobatidis* infection load, the cause of chytridiomycosis, was positively correlated with pH on the ventral, but not the dorsal, skin of frogs which may be a cause or a consequence of infection (Woodhams *et al.*, 2012).

Skin pH is genetically determined to a degree, but is also affected by behavior and environment (Sakuma and Maibach, 2012). Factors influencing skin pH include: 1. endogenous factors such as age, anatomical location, genetic predisposition, amount of melanin in skin, glandular secretions (sebaceous, apocrine, eccrine), and moisture; and 2. exogenous factors such as topical products, occlusive dressings, and skin irritants (e.g., various chemicals; Matousek and

Campbell 2002; Schmid-Wendtner and Korting 2006). Mouse skin is largely acidified by endogenous agents, such as the sodium-proton antiporter (NHE1) and secretory phospholipase A_2 ($sPLA_2$)-mediated extracellular generation of free fatty acids from phospholipids (Behne *et al.*, 2002; Fluhr *et al.*, 2004). Research on humans, laboratory, and domestic mammals shows that skin pH varies with season (human skin most acidic in July), body part, sex, age, species, and breed in domestic mammals (Byrd *et al.* 2018; Chikakane and Takahashi 1995; Matousek and Campbell 2002; Meyer and Neurand 1991). Skin pH of wildlife has rarely been studied (Table 4.S1), but does include data for various bird species and naked mole rats (*Heterocephalus glaber*) in zoos (Bartels *et al.*, 1991; Menon *et al.*, 2019), and amphibians and fish in laboratories (Tsui *et al.*, 2002; Litwiller *et al.*, 2006; Woodhams *et al.*, 2012; Barnhart *et al.*, 2020). Skin pH has not yet been quantified in free-ranging populations but may be an important component in assessing both inter- and intraspecific responses to infectious pathogens.

Skin diseases of wildlife have received increasing attention over the past few decades (Fisher *et al.*, 2016). The best-known skin disease of bats is white-nose syndrome (WNS), a cutaneous infection caused by the fungal pathogen *Pseudogymnoascus destructans* that damages flight membranes during hibernation and can lead to starvation, dehydration, and death (Lorch *et al.*, 2011a; Cryan *et al.*, 2013). The disease varies seasonally and variation in host susceptibility has been documented both among and within species (Frank *et al.* 2014; Langwig *et al.* 2012; Turner *et al.* 2011). Some Nearctic bat species have experienced catastrophic population declines due to WNS and are now listed as endangered (Solari, 2018). Previous research on the ability of *P. destructans* to use various nutrient sources, secrete enzymes, and interact with other microbes conducted experiments at various pH levels, without knowing the pH of bat skin (Beekman *et al.*, 2018; Donaldson *et al.*, 2018; Gabriel *et al.*, 2019; Vanderwolf *et al.*, 2021a). Cultures of *P.*

destructans grow *in vitro* from pH 4 to 11 (Raudabaugh and Miller, 2013; Vanderwolf *et al.*, 2021a), although a carboxypeptidase enzyme produced by the fungus *in vitro* was most active at 3 – 5 pH compared to 6.5 – 8.5 pH (Beekman *et al.*, 2018). Cultures of *P. destructans* alkalinize some growth media *in vitro* (e.g. ~5.6 to 7.9 pH) (Veselská *et al.*, 2020), but it is unknown if the fungus alkalinizes bat skin. Prior to WNS, skin diseases were not commonly reported in bats, although dermatophytes are known to grow on bat skin (Simpson *et al.*, 2013; Lorch *et al.*, 2015; McAlpine *et al.*, 2016) and dermatitis has been documented (Goodnight, 2015; Fountain *et al.*, 2017, 2019). A global survey of captive bats found that some species are more frequently reported with skin diseases compared to others, and some skin lesions show seasonal patterns with increased frequency in the winter for bats exposed to outdoor temperatures (Fountain *et al.*, 2017).

Given the strong link between skin chemistry and susceptibility to cutaneous diseases demonstrated in humans and domestic mammals, variation in skin chemistry may partly explain why bats vary in susceptibility to skin diseases such as WNS. Inter- and intra- specific or seasonal variation in bat skin pH may partially explain corresponding variation in cutaneous microbiomes and responses to pathogens. We measured the skin pH of five bat species at 32 locations across eastern Canada over one year to determine how flight membrane pH differs with species, season, body part, sex, age-class, geographic location, and pH of roosting substrates. Skin pH of humans varies among body parts and seasons (Abe *et al.*, 1980; Schmid-Wendtner and Korting, 2006; Wan *et al.*, 2014), and we hypothesized that similar mechanisms apply to bats, predicting that we would observe the most acidic skin pH in summer months. Previous research on humans and domestic animals found sex-based variation in skin pH and therefore we predicted there would be variation in skin pH between sexes in bats. However, the direction of

the sex effect varied among species and studies (Jenkinson and Mabon, 1973; Ruedisueli *et al.*, 1998; Giacomoni *et al.*, 2009; Szczepanik *et al.*, 2011), so we could not predict the direction of the effect in bats. Finally, while we could not make directional predictions about site-specific variation in bat skin pH, we expected that roost site characteristics might affect bat skin pH, predicting that skin pH might vary among capture locations. Data on the skin pH of bats will inform future research into the functionality of enzymes on the skin surface. Our interest in this topic stems from the potential link between skin chemistry and disease susceptibility. The bats we measured in this study have all survived multiple years with WNS, meaning that our study populations of "susceptible" *M. lucifugus* have already undergone selection for tolerance or resistance to WNS (Donaldson *et al.*, 2017; Cheng *et al.*, 2019; Auteri and Knowles, 2020). Therefore, we did not attempt to correlate skin pH directly with species' susceptibility to WNS. Instead, our study provides a baseline for further work on disease susceptibility and potential treatments for skin diseases of bats.

METHODS

We caught wild bats in eastern Canada at: 1) maternity colonies, where bats give birth and raise pups (May – mid-July 2019), 2) swarming sites, where bats congregate and mate at potential hibernacula such as caves and mines (mid-July – October 2019), and 3) hibernation sites, where bats overwinter in underground structures (February 2020) (Figure 4.1). Bats at maternity and swarming sites were caught using mist nets and harp traps, while bats at hibernation sites (caves and mines) were caught by hand from the walls and ceilings. Maternity colonies were in bat boxes, attics, and the siding of buildings. Bats were caught outside caves and mines during swarming season. There was temporal overlap of measurements taken of wild

bats in Atlantic Canada and Ontario during maternity and swarming seasons. Wild bats were only measured in February during the hibernation period to minimize disturbance, and gloves were changed between processing each bat to minimize microbial transfer. We recorded the species, sex, weight, and age (juvenile or adult) of each bat (OMNRF WACC authorization #19-394; Trent University animal care authorization 26117, New Brunswick Species At Risk permit #SAR19-014). Species included *Eptesicus fuscus, Myotis lucifugus*, *M. leibii, M. septentrionalis,* and *Perimyotis subflavus.* We distinguished young-of-the-year from adults by examining the degree of fusion of the epiphyseal growth plates of the phalanges in July and August (Kunz and Anthony, 1982), however some young-of-the-year were likely classified as adults during swarming season. We were unable to differentiate age-classes further in our study, but follow-up work could also record whether testes were descended, to further separate young-of-the-year from adults. Bats were released on site after we completed measurements. Field work was only conducted on nights with no rain in the interests of bat welfare.

We measured skin pH using a pH meter (PH905; Courage and Khazaka Electronic GmbH, Mathias-Brüggen-Str. 91 50829 Köln, Germany) that attaches to a multiprobe adapter system (MPA2; Courage and Khazaka Electronic). The probe measures surface pH and does not penetrate the skin. The diameter of the sensor was 1 cm. We took three consecutive measurements per skin site with the pH meter and used the mean as the final value. We repeated measurements if the three measurements from one skin site differed by more than 0.2 pH, as large variation indicates the probe was held incorrectly. Since skin pH varies among body parts in humans (Schmid-Wendtner and Korting, 2006), we quantified fine-scale variation in skin pH across the flight membrane by taking measurements of 38 sections (in a grid pattern) on the right wing and tail membrane from a subset of bats (Figure 4.2; n = 4 individuals). Based on these

initial results (Figure 4.2), and to standardize measurements among individual bats and investigate variation in pH among body parts, we subsequently took three measurements ('arm', 'plagiopatagium', and 'uropatagium'; Figure 4.2) on the dorsal side and three measurements on the ventral side of the right wing and tail membrane. We stored the end of the pH probe in KOH and washed it in distilled water between each set of measurements, as recommended by the manufacturer. We calibrated the pH probe every day for the first month and thereafter once a week with 4 and 7 pH buffers, exceeding the manufacturer recommendation of calibration every three weeks.

Initially, we also attempted to measure the amount of sebum on the surface of bat flight membranes with a sebumeter (SM815; Courage and Khazaka Electronic GmbH, Mathias-Brüggen-Str. 91 50829 Köln, Germany). However, this probe was designed for use on humans and, from our initial observations, was not sensitive enough to detect small amounts of sebum on bat flight membranes. Many of our measurements of bat skin using the sebumeter were zero. Non-zero values were not reproducible and appeared to be affected by the presence of urine on the skin.

To investigate temporal variation in skin pH, we measured an *E. fuscus* captive research colony at McMaster University (Hamilton, Ontario, Canada) monthly from April 2019 to March 2020. The colony has two living areas: an "Established" side and a "Quarantine" side. The Established side houses bats that have passed quarantine, whereas the Quarantine side houses recently captured wild bats that stay in quarantine a minimum of three months after arriving, as well as bats that have been in the colony for months or years but are being used in current experiments. Bats in the Established colony have year-round access to an outdoor flying area (Skrinyer *et al.*, 2017). Bats on both sides of the colony have constant access to water, meal

worms (*Tenebrio molitor*, Reptile Feeders, Norwood, ON) and space allowing them to fly. The colony temperature and lighting vary with ambient conditions; however, both indoor living areas are buffered from ambient temperatures, particularly in the winter (Figure 4.S1). Daily maximum and minimum temperature and humidity in the captive colony was measured with an Acurite indoor/outdoor digital thermometer and hygrometer (model # 00219CA). The captive bats typically roost in between and behind layers of cotton bath towels folded in half and hung on the colony walls, and some bats roosted inside two wooden structures. To investigate possible influences of roost pH on skin pH we also measured the pH of each layer of the four towels (1 outside layer, 3 inner layers of each towel; n=3 measurements for each layer) monthly from December – March 2019. We measured the inside surface pH of the wooden roosts once in February 2019.

We conducted a literature review on the skin pH of animals to put our data in the context of previous studies. We located papers using the internet search engines Thomson Reuters' ISI Web of Science and Google Scholar, as well as by scanning bibliographies of relevant papers, on May 3, 2021 using the keywords 'wildlife "skin pH"' and 'animal "skin pH"'. Conference abstracts and posters were excluded and only studies on intact animals were included (i.e., *in vitro* studies of tissue samples were excluded).

Data Analysis

Unless stated otherwise, data are presented as the mean ± standard deviation (SD). We performed all statistics in R (R Core Team, 2020). We constructed all graphs using ggplot2 (Wickham, 2016). Data used to construct Figure 4.2 was interpolated using the function 'idw' in the gstat package (Graler *et al.*, 2016) in addition to using ggplot2, raster, scico, and sf packages

(Wickham, 2016; Pebesma, 2018; Pedersen and Crameri, 2020; Hijmans, 2021). We used linear mixed effects models (package 'lme4'; (Bates *et al.*, 2015) to determine which variables affected bat skin pH in three separate models for the capture seasons: maternity (May – July), swarming (August – October), and hibernation (November – April). We set the individual bat as a random effect in each model (six measurements taken per bat) to control for inter-individual variation. Fixed effects potentially affecting skin pH included intrinsic (sex, species, age, body part, flight membrane surface) and extrinsic factors (day of year and site). 'Membrane surface' refers to the dorsal and ventral sides of the flight membranes, and 'body part' refers to the three flight membranes that were measured: arm, plagiopatagium, and uropatagium. We did not include age (juvenile, adult) in the hibernation model because young of the year cannot be differentiated from adults during winter. We also did not include day-of-year in the hibernation model because the skin pH of wild bats during the hibernation period were measured over a 10-day period in February. Additionally, we used generalized additive mixed models with individual bat as a random effect using the packages 'mgcv' and 'MuMIn' (Wood, 2017; Barton, 2019) to determine the impact of the fixed effects previously listed on skin pH for three species (*E. fuscus* [both captive and wild-caught], *M. lucifugus,* and *M. leibii*). We applied a smoothing factor to day-of-year for each bat species. We added maximum and minimum temperature and relative humidity (on the measurement day) as fixed effects to the model for captive *E. fuscus*. We excluded *M. septentrionalis* from statistical analyses given low sample size (n = 4 individuals). We used a linear mixed effect model for *P. subflavus* because this species was sampled over a limited timespan. We used the function AICtab (package bbmle) (Bolker and Team, 2017) to compare Akaike information criteria (AIC) values to determine the best model. Including 'region' (Ontario, Quebec= region 1, Maritime provinces= region 2) as a fixed effect in the

models for maternity season, swarming season, wild *E. fuscus*, and *M. lucifugus* did not improve the models. Models with 'region' in place of 'site' were inferior. Region was not included in models for hibernation, *P. subflavus*, captive *E. fuscus*, and *M. leibii* because measurements were obtained in only one region. We compared the skin pH of captive and wild *E. fuscus* with a generalized additive mixed model (smoothing factor applied to day-of-year), with captive status, day-of-year, sex, body part, and membrane surface as fixed effects and individual bat as a random effect. We tested for intra-individual associations of skin pH among the six body parts measured using the captive colony dataset with a repeated measures correlation in the package 'rmcorr' (Bakdash and Marusich, 2020). We tested whether the rank order of captive individual bats was consistent across twelve months of sampling by calculating the intraclass correlation coefficient using the package 'irr' (Gamer *et al.*, 2019) with a one way model, inter-rater agreement, and the mean skin pH of the six body parts for each individual in each month.

RESULTS

We measured 710 wild bats comprising five species (Table 4.S2). The range in skin pH was 4.67 – 8.50 for *M. lucifugus* (n = 528 individual bats), 5.48 – 8.42 for *M. leibii* (n = 28), 6.36 – 7.88 for *M. septentrionalis* (n = 4), 5.83 – 8.59 for *P. subflavus* (n = 19), 4.97 – 8.17 for wild *E. fuscus* (n = 131), and 4.40 – 7.80 for captive *E. fuscus* (n = 678 measuring sessions for 126 individual bats). Skin pH varied among species and on average *E. fuscus* was the most acidic across all three seasons (maternity, swarming, and hibernation), although there was no significant pH difference between wild *E. fuscus* and *M. leibii* during hibernation (Table 4.1, Figure 4.3).

Bats were most acidic in July in both the captive *E. fuscus* colony (5.1 ± 0.3 pH in July) and wild bats (6.0 ± 0.5 pH in July for all species at all sites), but note that wild bats were not measured from November – April, except for February (Figure 4.4). Although wild bats were measured at multiple sites, the standard deviation among wild bats was similar to that observed in the captive colony (0.5 vs. 0.3). This suggests that the time-series of the captive bats' skin pH provides a meaningful benchmark for temporal trends in skin pH of wild bats, despite colony-specific variation that may be associated with different roosting substrates or diet. The skin pH of wild bats had large seasonal variations, while seasonal patterns in the captive *E. fuscus* colony were more attenuated (Figure 4.4). Skin pH significantly decreased over the maternity season and increased over the swarming season in both wild and captive bats (Table 4.1, Figure 4.4). The skin pH of captive *E. fuscus* gradually increased from the beginning of hibernation season, peaked in February (6.4 ± 0.5 pH), and then gradually decreased towards spring (Figure 4.4). Changes in skin pH over the hibernation season could not be assessed for hibernating wild bats since they were exclusively measured in February (8.1 ± 0.3 pH for four species of wild bats in February).

Geographic site also influenced skin pH, although its effect was dependent on the time of year each site was sampled (Table 4.1, Figure 4.5). For example, we measured *M. lucifugus* at site ON16 early and late in the swarming season and skin pH increased over this period (Figure 4.S2). Similarly, we measured both *E. fuscus* and *M. lucifugus* at sites ON5 and ON8 early and late in maternity season and skin pH decreased over this time (Figure 4.S2).

There was no significant difference in skin pH between wild juvenile and adult bats during swarming, but juveniles were more acidic than adults in the maternity season model and in the *E. fuscus* and *M. lucifugus* species models (Table 4.1, Figure 4.6). Although all juveniles

included in the statistical analysis were volant, two adult females were caught carrying non-volant pups during the maternity season in New Brunswick. A female *E. fuscus* caught July 3, 2019 carrying a furless pup had a mean of 5.6 pH (range: 5.45 – 5.64) for the six standard skin measurements, while the pup had a mean of 5.15 pH on its back. A female *M. lucifugus* caught July 11, 2019 was carrying a furred male pup which measured 6.2 pH on the lower back, while the mother's mean for the six standard skin measurements was 6.1 pH (range: 6.04 – 6.22).

Wing-skin of male *M. lucifugus* and captive *E. fuscus* were more acidic than females during maternity season (and during late hibernation in captive bats), but this trend reversed during swarming (and early hibernation in captive bats) (Figure 4.4). Wild *E. fuscus* males were more alkaline than females during maternity season. There were no sex differences in skin pH from wild bats during hibernation or in *M. leibii* and *P. subflavus* (Table 4.1, Figure 4.3).

The pH of individual bats was not constant over time. The skin pH of five wild bats caught twice, and two wild bats caught three times, varied over time, with different patterns among individuals (Figure 4.S3). The skin pH of individual captive *E. fuscus* also varied temporally, and there was agreement among months in the rank order of individual bats with respect to mean skin pH ($F_{1,22} = 0.478$, $p = 0.497$), implying the impact of external factors (Figure 4.7).

In wild bats, mean pH range among body parts of an individual was 0.60 ± 0.28 (range: 0.11 – 1.78; Figure 4.S4), while in captive bats it was 0.78 ± 0.27 (range: 0.22 – 1.84). Dorsal flight membranes were more acidic than their ventral surfaces during maternity season, particularly in females (Table 4.1, Figure 4.3). This pattern reversed during swarming and hibernation seasons as the ventral surface was more acidic in wild bats, particularly for males during swarming. Wing sites closest to the body were more acidic than those further from the

body, and the ventral uropatagium was particularly acidic (Figure 4.2). The plagiopatagium was the most alkaline flight membrane and the uropatagium was the most acidic during swarming in all bat species except wild *E. fuscus*. The arm was most acidic in wild *E. fuscus* and during maternity season. Skin pH did not differ among body parts during hibernation in wild bats, although differences persisted in captive *E. fuscus* (Figure 4.3). Skin pH for the six standard flight membrane locations were highly correlated with each other within an individual over time in captive bats (Table 4.2).

Captive *E. fuscus* (Model estimate=5.6 ± 0.02, p<2e-16) had more acidic skin than wild *E. fuscus* (Model estimate=6.4 ± 0.04, p<2e-16; Figure 4.4). The maximum and minimum temperature and relative humidity were dropped as explanatory variables from the best model for the skin pH of captive *E. fuscus*, indicating they explained little to no variation in skin pH. The pH of the four towels measured over four months in the captive colony was 6.0 ± 0.6 for the outer layer and 5.6 ± 0.4 for the three inner layers (range: 4.5 pH for the inner layers to 6.89 pH for the outer layer). The inside lid and walls of the wooden roost structure in the established captive colony were 6.7 pH and 6.6 pH, respectively. In contrast, the inside lid and walls of a similar wooden roost structure in the relatively little-used quarantine side of the colony measured 7.3 pH and 8.0 pH, respectively. The roosting towels in the established side of the captive colony were replaced with clean towels twice over the study period: first, a month before measurements were recorded in June 2019, and again a week before the November 2019 measurements. These towel changes correlated with an increase in skin pH of individual captive bats (Figure 4.7).

A summary of previous literature on the skin pH of non-human vertebrates is presented in Table 4.S1. Several methodological details were sometimes missing from papers, particularly the

time of year measurements were taken. Previous studies exclusively measured captive or domestic animals, with dogs and laboratory mice the most commonly studied.

DISCUSSION

We investigated variation in the skin pH of bats to provide a baseline for future research on the association between skin chemistry and cutaneous infection in bats and other wildlife, exploring how skin pH varied among species, body part, season, sex, age class, and sampling location. Among the species we measured, *E. fuscus* had the most acidic skin and *M. septentrionalis* had the most alkaline skin (Figure 4.3). While we could not explicitly test the link between skin pH and WNS susceptibility, it is intriguing that the five species' skin pH fell along the same spectrum as their predicted susceptibility to WNS. Skin pH also varied between the sexes, by season (most acidic in July), and among body parts, which is consistent with previous studies on the skin pH of humans and domestic mammals (Byrd *et al.* 2018; Chikakane and Takahashi 1995; Matousek and Campbell 2002; Meyer and Neurand 1991). The direction of the sex effect changed between the maternity and swarming season for *M. lucifugus* and *E. fuscus*. The pH of bat flight membranes also varied by age class and sampling location (Table 4.1).

Our study is the first to measure skin pH in free-ranging vertebrates. Comparing our results with previous research on skin pH is difficult because seasonal variation has only been studied in humans, and most studies did not report which months measurements were taken (Table 4.S1). Additionally, some studies measured the fur/hair of animals instead of directly measuring the skin, although the fur/hair was shaved or clipped prior to measurement in some investigations. Nevertheless, our measurements of bat skin pH overlap with those from domestic mammals, except for some very alkaline (> 9 pH) values in domestic sheep, (likely because the

wool was measured and not the skin; Table 4.S1). In humans, skin pH has a circadian rhythm in some, but not all, body parts and can vary ~ 0.3 pH, with maximal values in the afternoon (14:00 - 16:00) and minimal values in the evening (~ 20:00; Yosipovitch *et al.*, 1998). We measured captive bats during the day and wild bats during the night, except during hibernation when wild bats were also measured during the day, hence circadian rhythms may explain some of the variation we documented in bat skin pH.

We found that skin pH varied among bat species (Table 4.1, Figure 4.3), which may be caused by multiple factors. Diet varies among the insectivorous bat species we studied. For example, *E. fuscus* may be beetle specialists (Thomas *et al.*, 2012) and captive *E. fuscus* in our study were exclusively fed meal-worms (i.e. Tenebrionid larvae). In humans, there is contradictory evidence for the effect of diet on skin pH (Prakash *et al.*, 2017; Lim *et al.*, 2019), and skin pH in cattle and cats did not vary with diet (Jenkinson and Mabon, 1973; Bourdeau *et al.*, 2004). However, sebum can be affected by diet (Lovászi *et al.*, 2018). Sebum quantity and skin pH are inversely correlated in humans (Wan *et al.*, 2014), and bat flight membranes have sebaceous glands which vary in abundance by species (Cortese and Nicoll, 1970; Sokolov, 1982; Yin *et al.*, 2011). The composition and quantity of fatty acids that comprise sebum on bat flight membranes also varies among species and seasons (Pannkuk *et al.*, 2012; Frank *et al.*, 2016) and could affect skin pH. This may influence or be influenced by seasonal variation in skin pH given that enzyme activity in epidermal tissue, which produce fatty acids, is pH-dependent (Behne *et al.*, 2002; Fluhr *et al.*, 2004). Some free fatty acids are generated within skin from phospholipids by secretory phospholipase A_2, and this enzyme is inactivated at alkaline pH (>7 pH), partially due to the activation of serine proteases (Behne *et al.*, 2002; Fluhr *et al.*, 2004). The total fatty acid content of bat wing skin decreases over hibernation (Frank *et al.*, 2016), and we found that

the skin of hibernating bats is typically alkaline. However, the skin pH of young laboratory mice with sebaceous gland hypoplasia did not differ from wild-type mice, suggesting minimal effect of sebaceous gland products on the development of adult acidic skin pH from the neonatal alkaline state (Fluhr *et al.*, 2004). The acidification of neonate skin starts in deeper layers and moves upwards to the surface, and a pH gradient also exists in adults as deeper layers are more acidic compared to the skin surface (Behne *et al.*, 2002, 2003; Fluhr *et al.*, 2004). This highlights the importance of endogenous skin processes in maintaining an acidic skin pH, such as the activity of the sodium-proton antiporter and secretory phospholipase A_2 (Behne *et al.*, 2002, 2003; Fluhr *et al.*, 2004). We acknowledge that humans and laboratory mice may not be the most relevant model systems for understanding skin chemistry in wild bats, but these studies provide evidence for drivers of skin pH, allowing us to generate testable hypotheses for future research in wild mammals. We also acknowledge that we only measured the surface pH of bat skin, and that a pH gradient may exist within bat wing tissue like that observed in mice and humans. However, dermal and hypodermal layers of bat wings are greatly reduced compared to typical mammalian skin (Sokolov, 1982), suggesting lower variation than in other mammals. Finally, the current study does not allow us to untangle the associations among sebum, skin pH, and diet in bats, which should be further investigated.

In humans, cutaneous pH varies among body parts, and occluded areas (e.g., axillae, genitoanal region, submammary folds, and interdigital areas) are generally more alkaline (6 – 7 pH) than drier areas (4 – 6 pH) (Schmid-Wendtner and Korting, 2006). Skin pH also varies among body parts in domestic mammals (Jenkinson and Mabon, 1973; Mok *et al.*, 1982; Meyer and Neurand, 1991; Ruedisueli *et al.*, 1998; Proksch, 2018). Roosting bats fold their wings, which may increase moisture and lead to higher pH levels. The uropatagium may be more acidic

than the wing in bats (Figure 4.3) because of repeated exposure to urine, an acidic liquid. The urine pH of the bat species we studied may be as acidic as *Myotis velifer* urine (mean 5.5 – 6.0 pH, range: 5.1 – 9.4 pH; depending on month) (Shackelford and Caire, 1993), or that of five European bat species (mean 5.3 – 6.8 pH) (Hales, 2014). In humans, grooming habits affect skin pH. For example, showering temporarily increases skin pH (Lambers *et al.*, 2006). Grooming (i.e. licking) skin may temporarily affect bat skin pH as *M. lucifugus* and *M. septentrionalis* saliva is ~7.5 – 8.5 pH (Dumont, 1997), and may partially explain why different body parts varied in pH if bats do not groom all areas equally. However, the pH of the roosting environment may also affect skin pH. Dorsal flight membrane surfaces may have been more acidic than ventral surfaces year-round in the captive *E. fuscus* colony because roosting substrates are acidic year-round (except for a short period after roost towels are cleaned/replaced). Roosting substrates in the captive *E. fuscus* colony were likely acidic due to the accumulation of body wastes, especially urine, and new roosting towels became noticeably stiffer with time due to saturation with dried urine. This suggests interior surfaces of natural maternity roosts are also acidic due to accumulation of nitrogenous waste, and dorsal flight membranes were more acidic than ventral surfaces during maternity season. During swarming and hibernation wild bats roost on cave walls which are generally alkaline (7 – 8 pH) (Hajna, 2003; Shahack-Gross *et al.*, 2004; Portillo and Gonzalez, 2010), and their dorsal flight membranes were more alkaline than ventral surfaces during this time. Our findings indicate that roosting substrates within bat colonies may influence skin pH and therefore possibly skin function, which is relevant to experiments involving captive bat colonies.

The pH of roosting substrates affects skin pH and may partially explain the seasonal patterns we observed (Figure 4.4), as bats switch roosts from one season to the next. However,

we also documented seasonal variation in skin pH in the captive *E. fuscus* colony, where bats live in the same enclosure and therefore urinate on the same roosting substrates year-round. Seasonal variation in skin pH has also been documented in humans, many of which do not change dwellings seasonally (Abe *et al.*, 1980; Nakagawa *et al.*, 2004; Wan *et al.*, 2014). Humans are most acidic in July and most alkaline in January, although subjects were only measured four months of the year (Abe *et al.*, 1980). The mean seasonal change in human skin pH is 0.4 – 1.5 from summer to winter (Abe *et al.*, 1980; Nakagawa *et al.*, 2004; Wan *et al.*, 2014), while we documented a mean change of 2.1 and 1.4 pH from July to February in wild and captive bats, respectively. The larger seasonal variation in skin pH of wild bats is likely related to the effects of hibernation, exposure to outside temperatures, and changes in roosting substrates. Future studies should consider repeated measures from wild maternity colonies throughout the active season to help untangle the effects of site and season on skin pH.

Ambient temperature and humidity may partially drive seasonal changes in skin pH indirectly by affecting sweat and sebum production. We did not detect an effect of temperature or relative humidity on the skin pH of captive *E. fuscus*, likely because these bats were somewhat buffered from the seasonal changes in weather experienced by wild bats (Figure 4.S1). One hypothesis for low skin pH during summer in humans is increased eccrine sweat secretion stimulated by increasing skin temperature (Abe *et al.*, 1980). This explanation is unlikely to apply to bats as eccrine glands in non-human mammals are confined to footpads (Folk and Semken, 1991), and instead bats evaporatively cool by panting, or licking and fanning their wings (Baudinette *et al.*, 2000). Sweat glands in bats are reported as either absent (Sokolov, 1982; Makanya and Mortola, 2007) or exclusively apocrine (Sisk, 1957; Cortese and Nicoll, 1970). Sebum quantity and skin pH are inversely correlated in humans, and sebaceous gland

activity increases with increases in humidity and especially temperature (Sakuma and Maibach, 2012; Wan *et al.*, 2014).

We found a sex difference in skin pH among bats, but only during the active season (Table 4.1, Figure 4.3), possibly due to the use of different roost types by the sexes and/or hormonal variation. In many temperate insectivorous bats, the sexes largely segregate from early spring through mid-summer with females forming maternity colonies and males in bachelor groups (Kunz and Fenton, 2003). Skin pH is higher in males than females in dogs (Ruedisueli *et al.* 1998), cats (Szczepanik *et al.*, 2011), and cattle (Jenkinson and Mabon, 1973; Meyer and Neurand, 1991), although other studies on various domestic mammals found no difference between the sexes (Table 4.S1). In humans, there are conflicting results concerning which sex is more acidic (Giacomoni *et al.*, 2009).

Age affects skin pH as neonates (< 1 month) and elderly humans (> 60 years) have more alkaline skin than adults, as do neonate laboratory rats and calves (Ajito *et al.*, 2001; Fluhr *et al.*, 2004; Choi *et al.*, 2007; Chan and Mauro, 2011; Proksch, 2018). Rats attain adult skin pH levels about one week after birth (Fluhr *et al.*, 2004), and humans after about one month (Proksch, 2018). We found volant juvenile bats had more acidic skin than adults (adults could not be aged) during maternity season but not during swarming (Table 4.1, Figure 4.6). Potentially, this reflects the large amount of time juveniles spend in maternity roosts as these roosting substrates may be acidic due to waste accumulation (we only measured the pH of roosting substrates in the captive colony).

Variation in skin pH among species and individuals may impact susceptibility to skin diseases. For example, the relatively high skin pH of dogs (7 – 8 pH) compared to other domestic animals may partially account for the disproportionally high incidence of pyoderma (superficial

bacterial infection of hair follicles and surrounding skin) in dogs (Mason *et al.*, 1996). Studies in humans, dogs, laboratory mice, and rats indicate that experimentally decreasing skin pH with topical products can prevent or ameliorate some skin diseases and speed recovery from injury, but not in all circumstances (Matousek *et al.*, 2003; Fluhr *et al.*, 2004; Hatano *et al.*, 2009; Lee *et al.*, 2014; Nagoba *et al.*, 2015; Panther and Jacob, 2015). In bats with WNS, it is unknown if the fungal pathogen (*P. destructans*), causes fewer skin lesions on acidic versus alkaline skin. Our data show *E. fuscus* has the most acidic skin (Figure 4.3), and this species is also more tolerant of WNS than the other bat species we measured (Cheng *et al.*, 2021). Although *P. destructans* can grow *in vitro* from 4.5 – 11 pH (Raudabaugh and Miller, 2013; Vanderwolf *et al.*, 2021a), a carboxypeptidase enzyme produced by *P. destructans* was most active at 3 – 5 pH compared to 6.5 – 8.5 pH *in vitro* (Beekman *et al.*, 2018). The skin of wild bats during hibernation varied from 6.2 – 8.6 pH, suggesting activity of this enzyme may be limited on the hibernating bats we measured. The activity of other potential virulence factors produced by *P. destructans*, the activity of bat skin defenses such as cutaneous antimicrobial peptides, and potential biological or chemical spray-on treatments for WNS should be assessed at pH levels representative of the skin of hibernating bats of different species. For example, some yeasts commonly cultured from bat wings inhibit *P. destructans in vitro*, but only at 4 – 5 pH and not 7 pH (Vanderwolf *et al.*, 2021a). This suggests that inhibition of *P. destructans* by these yeasts would not occur during hibernation on the skin of the bat species we measured during this study, since skin pH was > 7 pH during winter (Figure 4.3). However, pathogenic fungi can sense and respond to environmental pH, enabling survival, growth, virulence, and dissemination in different host niches by altering gene expression to produce enzymes that are functional at ambient pH (Martinez-Rossi *et al.*, 2017).

Future research on the influence of skin pH on the functionality of enzymes produced by microbes and bats may provide valuable insights on new therapeutic targets for treating bat skin conditions like WNS. Skin enzyme functionality is important in maintaining skin barrier function and for virulence factors produced by microbes. Skin pH may play a role in varying disease susceptibility among individuals and species by influencing enzyme functionality or the diversity of cutaneous microbiota. More data on skin pH in relation to other aspects of skin chemistry, and from more bat species in different geographic areas, may provide further insights on bat skin disease susceptibility.

TABLES AND FIGURES

Table 4.1**:** Results of linear mixed effects models with variables explaining flight membrane pH of wild bats at maternity, swarming, and hibernation sites. We performed generalized additive mixed models for each bat species, except for *Perimyotis subflavus* where we used a linear mixed effect model. Maximum and minimum temperature and relative humidity were dropped from the best model for captive *Eptesicus fuscus* and are not shown. The marginal (m) and conditional (c) R^2 are reported for linear mixed effect models, and the F-value with degrees of freedom and p-value are reported for each variable in each model. The two variables explaining the most variance in each model are in boldface type. NA = not applicable. D = dropped from the best model. NI = not included in the model.

Variable	Maternity	Swarming	Hibernation	Captive *Eptesicus fuscus*	Wild *E. fuscus*	*Myotis lucifugus*	*M. leibii*	*Perimyotis subflavus*
R^2	m=0.66, c=0.80	m=0.67, c=0.90	m=0.44, c=0.81	0.65	0.67	0.82	0.95	m=0.95, c=0.97
Site	F_{14}=31.3, p<2.2e-16	F_{12}=17.4, p<2.2e-16	F_2=6.3, p=0.004	NA	F_{13}=6.9, p=9.4e-13	F_{25}=13.6, p<2e-16	**F_6=210.2, p<2e-16**	**F_4=180.0, p=4.2e-13**
Species	F_2=31.4, p=7.2e-13	F_4=18.8, p=4.3e-14	**F_3=11.6, p=9.4e-6**	NA	NA	NA	NA	NA
Day of Year	F_1=48.5, p=8.2e-12	F_1=47.1, p=2.8e-11	NA	**$F_{11.0}$=651.3, p<2e-16**	$F_{8.4}$=9.1, p=1.2e-12	$F_{8.4}$=11.6, p=2.7e-11	**$F_{1.0}$=19.6, p=1.8e-5**	D
Sex	F_1=19.5, p=1.4e-5	F_1=4.1, p=0.044	F_1=0.1, p=0.703	F_1=7.2, p=0.007	F_1=14.7, p=1.3e-4	F_1=34.2, p=5.6e-9	D	D
Membrane Surface (dorsal/ventral)	**F_1=48.9, p=4.2e-12**	**F_1=225.7, p<2.2e-16**	F_1=5.5, p=0.019	F_1=178.9, p<2e-16	**F_1=49.0, p=5.6e-12**	F_1=66.9, p=4.2e-16	F_1=2.4, p=0.123	D

Body Part	**F_2=101.2, p<2.2e-16**	**F_2=466.4, p<2.2e-16**	F_2=5.3, p=0.006	**F_2=456.6, p<2e-16**	F_2=35.0, p=3.0e-15	**F_2=161.5, p<2e-16**	F_2=14.8, p=1.4e-6	**F_2=10.9, p=5.4e-5**
Age (Adult/Juvenile)	F_1=24.9, p=1.1e-6	D	NA	NA	F_1=5.0, p=0.025	F_1=7.1, p=0.008	NA	NA
Body Part* Wing Surface	F_2=7.4, p=6.2e-4	F_2=36.2, p=3.6e-16	F_2=3.4, p=0.036	F_2=58.2, p<2e-16	F_2=6.3, p=0.002	F_2=18.5, p=1.0e-8	F_2=2.5, p=0.09	D
Sex*Day of Year	F_1=17.3, p=4.0e-5	D	NA	NI	NI	NI	NI	D
Body Part*Day of Year	D	D	NA	NI	NI	NI	NI	D
Wing Surface*Day of Year	F_1=38.2, p=8.5e-10	D	NA	NI	NI	NI	NI	D
Sex*Body Part	D	D	D	D	D	F_2=5.4, p=0.005	D	D
Sex*Wing Surface	F_1=35.3, p=3.6e-9	F_1=30.2, p=4.4e-8	**F_1=6.7, p=0.010**	F_1=11.7, p=6.5e-4	**F_1=37.4, p=1.5e-9**	**F_1=199.6, p<2e-16**	D	D

Table 4.2: Repeated measures correlations for the six flight membrane sites measured in captive *Eptesicus fuscus*. Mean correlations (R_{rm}; in bold), the 95% confidence interval, and p-value are listed for each pairwise comparison. The degrees of freedom for each comparison was 591.

Variable	Dorsal Plagiopatagium	Dorsal Arm	Dorsal Uropatagium	Ventral Plagiopatagium	Ventral Arm	Ventral Uropatagium
Dorsal Plagiopatagium	1					
Dorsal Arm	**0.90**, 0.88:0.91, 1.07e-215	1				
Dorsal Uropatagium	**0.84**, 0.82:0.87, 9.9e-162	**0.91**, 0.90:0.93, 5.9e-233	1			
Ventral Plagiopatagium	**0.82**, 0.80:0.85, 4.4e-148	**0.85**, 0.83:0.87, 4.7e-167	**0.85**, 0.83:0.87, 5.3e-169	1		
Ventral Arm	**0.81**, 0.78:0.84, 5.6e-141	**0.84**, 0.82:0.86, 1.4e-160	**0.84**, 0.81:0.86, 4.7e-158	**0.95**, 0.94:0.96, 1.7e-304	1	
Ventral Uropatagium	**0.79**, 0.75:0.82, 4.3e-162	**0.86**, 0.84:0.88, 1.7e-178	**0.89**, 0.88:0.91, 4.7e-208	**0.86**, 0.84:0.88, 2.4e-173	**0.89**, 0.87:0.91, 5.1e-204	1

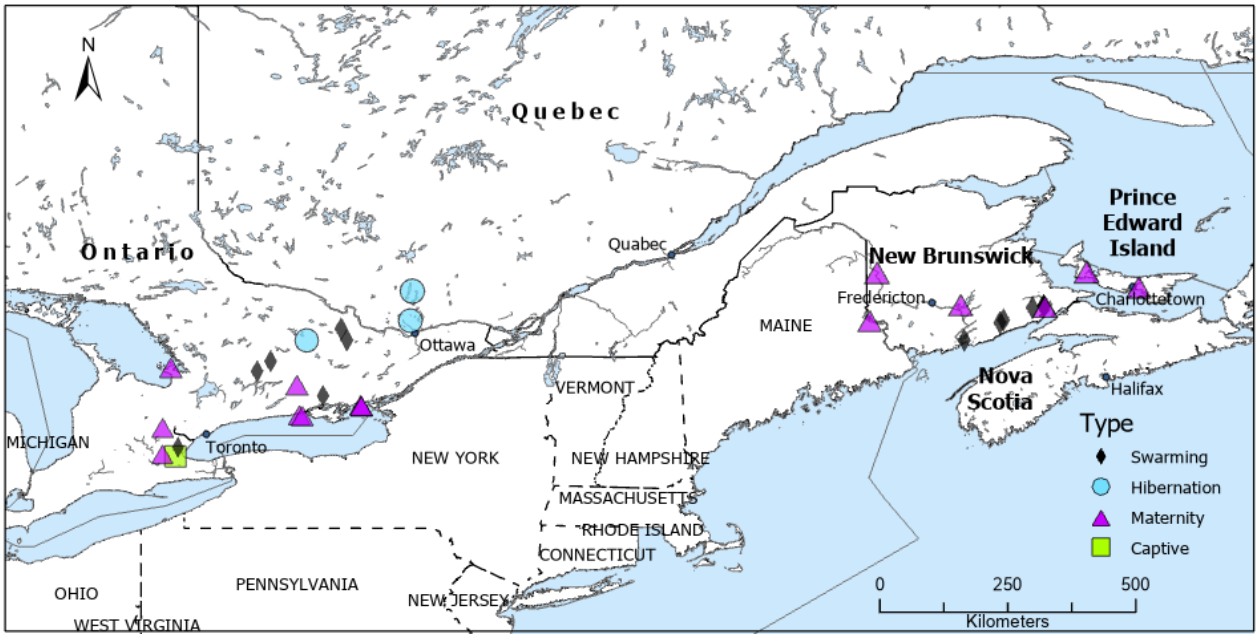

Figure 4.1: Sites where we measured the skin pH of bats at maternity (n = 15 sites, n = 270 individual wild bats), swarming (n = 13, n= 389), and hibernation sites (n = 3, n = 51) in 2019-2020.

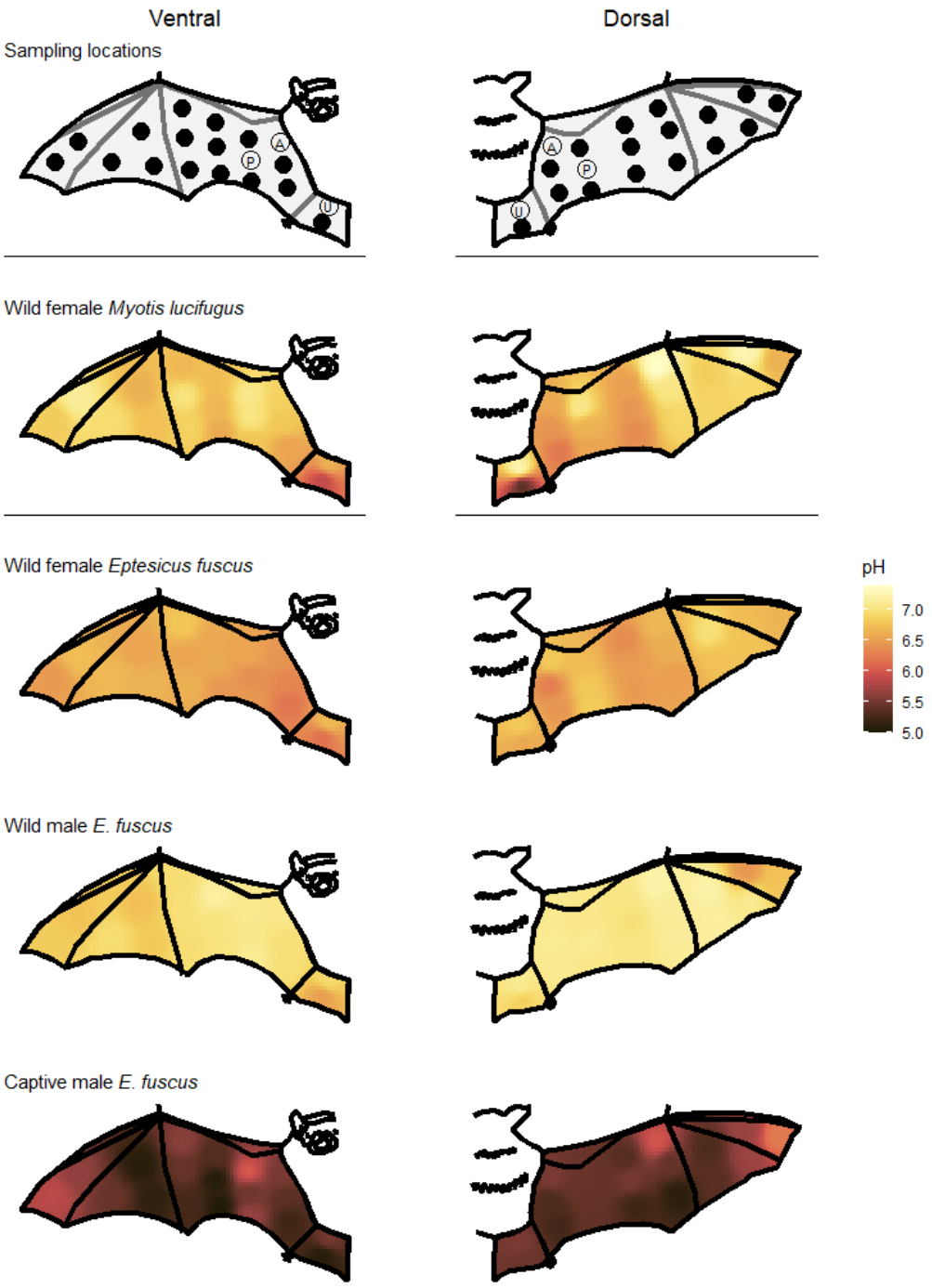

Figure 4.2**:** Schematic views of the right wing and tail membrane indicating where we measured skin pH. All 38 measurements were taken from 4 individual bats while 'P' (ventral and dorsal plagiopatagium), 'A' (ventral and dorsal arm), and 'U' (ventral and dorsal uropatagium) were taken from all bats. Heat maps illustrate skin pH measurements taken from the ventral (left; 19 skin sites per bat) and dorsal (right; 19 skin sites per bat) flight membranes of bats caught in Ontario 2019. The *Myotis lucifugus* and captive *Eptesicus fuscus* were measured in June, and the two wild *E. fuscus* were measured in May.

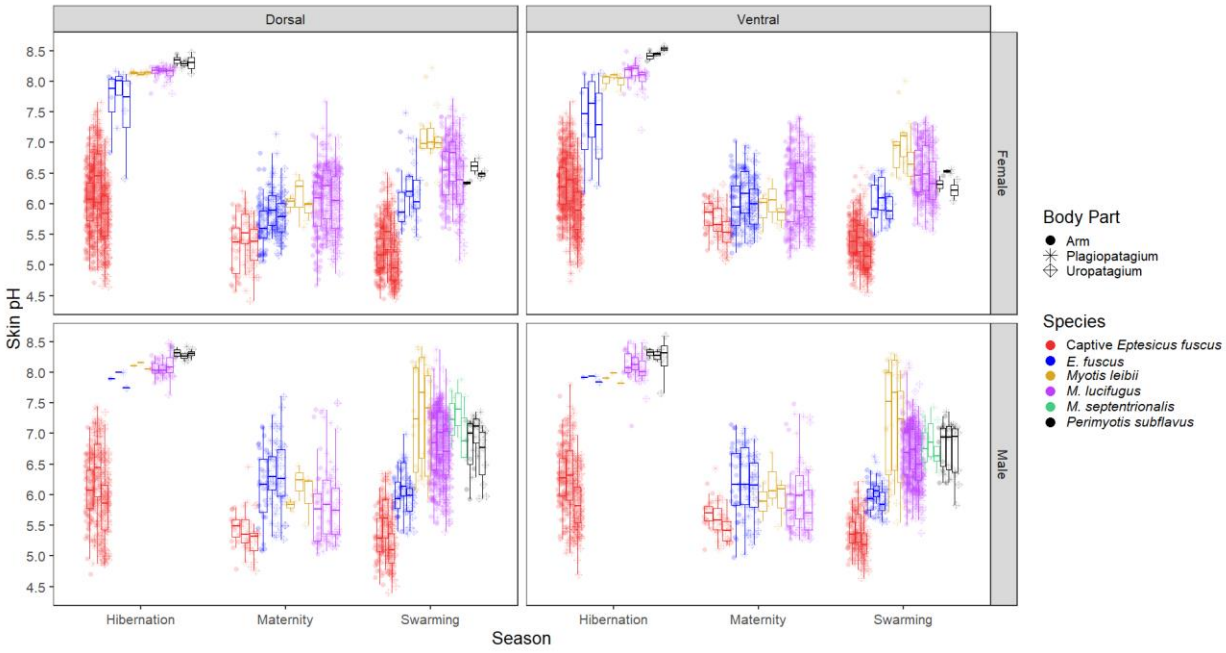

Figure 4.3**:** Box plots of flight membrane pH of captive *Eptesicus fuscus* and wild bats measured in Ontario, Quebec, New Brunswick, and Prince Edward Island, Canada, across three activity seasons: maternity (May – July), swarming (August – October), and hibernation (November – April for captive bats, wild bats only measured in February).

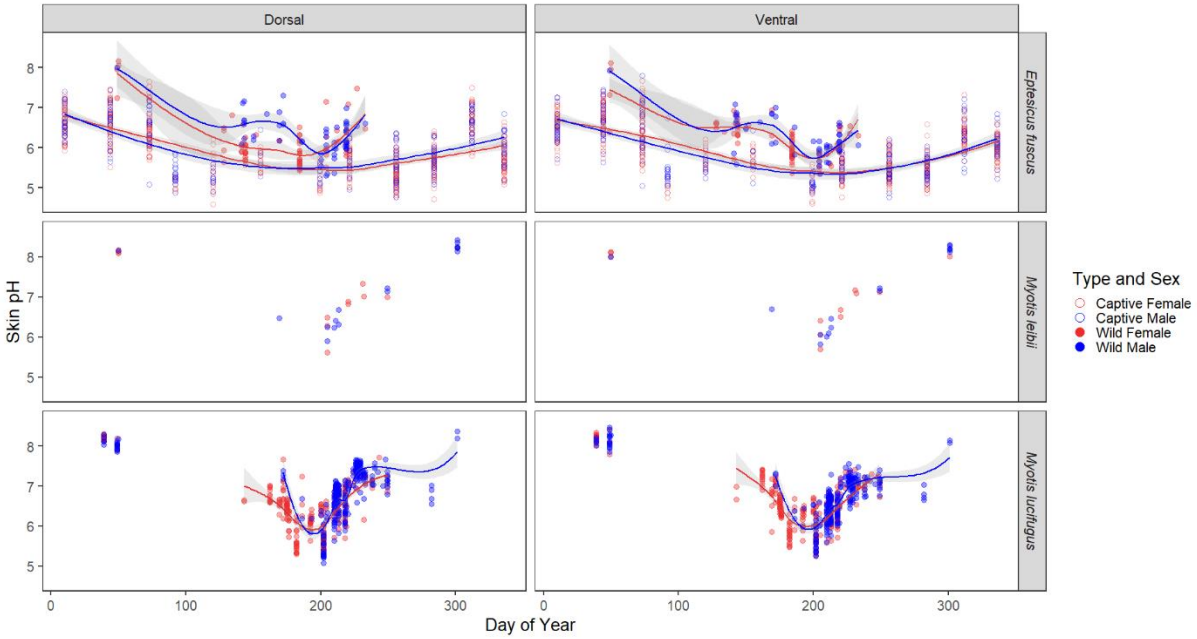

Figure 4.4**:** Plagiopatagium flight membrane pH of *Eptesicus fuscus* (captive and wild) and wild *Myotis leibii* and *M. lucifugus* over time (data from all provinces). Sample sizes are listed in Table S2. Lines indicating the mean pH (95% confidence intervals in gray shading) were predicted using the loess method. Seasonal patterns in *M. septentrionalis* and *Perimyotis subflavus* could not be assessed due to low sample sizes.

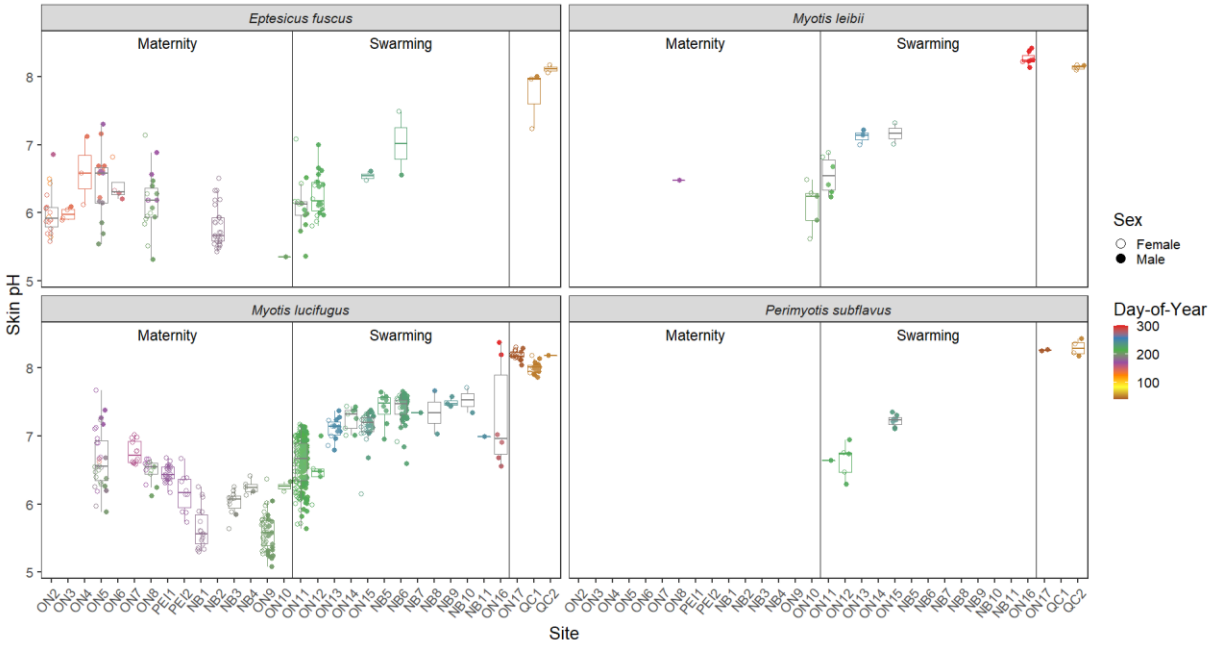

Figure 4.5**:** Dorsal plagiopatagium pH of wild bats at each geographical site. Sites are listed with their provincial abbreviation: ON=Ontario, NB=New Brunswick, PEI=Prince Edward Island, and QC=Quebec. Sites ON2 – ON10, PEI1 – PEI2, and NB1 – NB4 were measured during the maternity season. Sites ON11 – ON16 and NB5 – NB11 were measured during swarming season. Sites ON17, QC1, and QC2 were measured during the hibernation season.

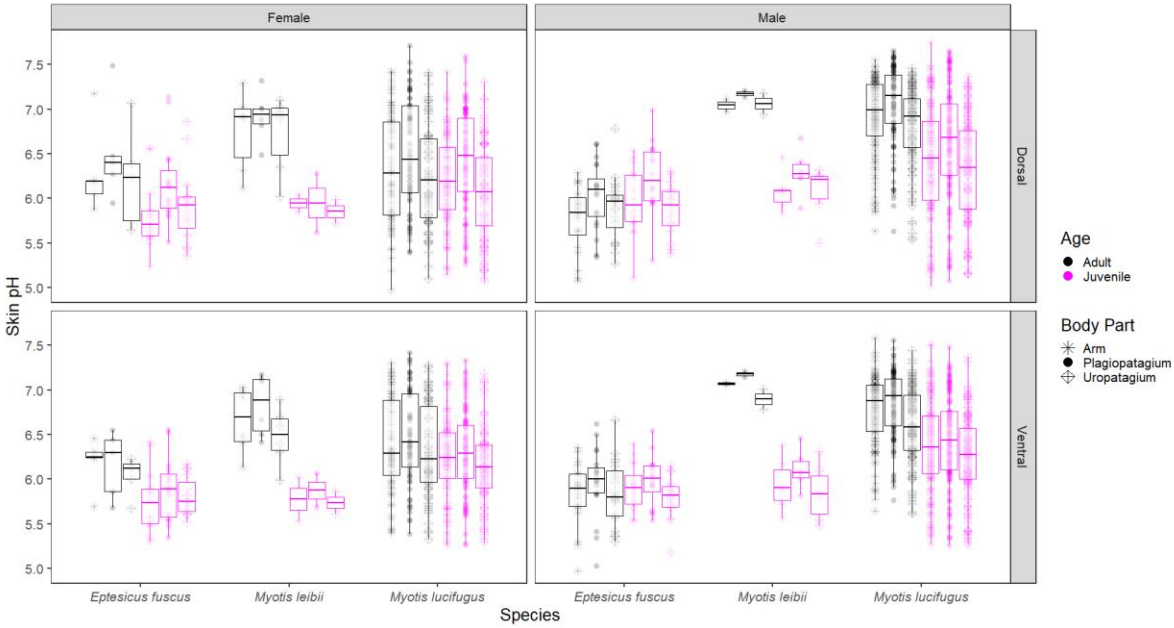

Figure 4.6**:** Wild volant juvenile and adult bats caught in Ontario and New Brunswick from day-of-year 186 – 250 (i.e. the first to last days that juveniles were caught). Note: we did not catch juvenile *Perimyotis subflavus* of either sex.

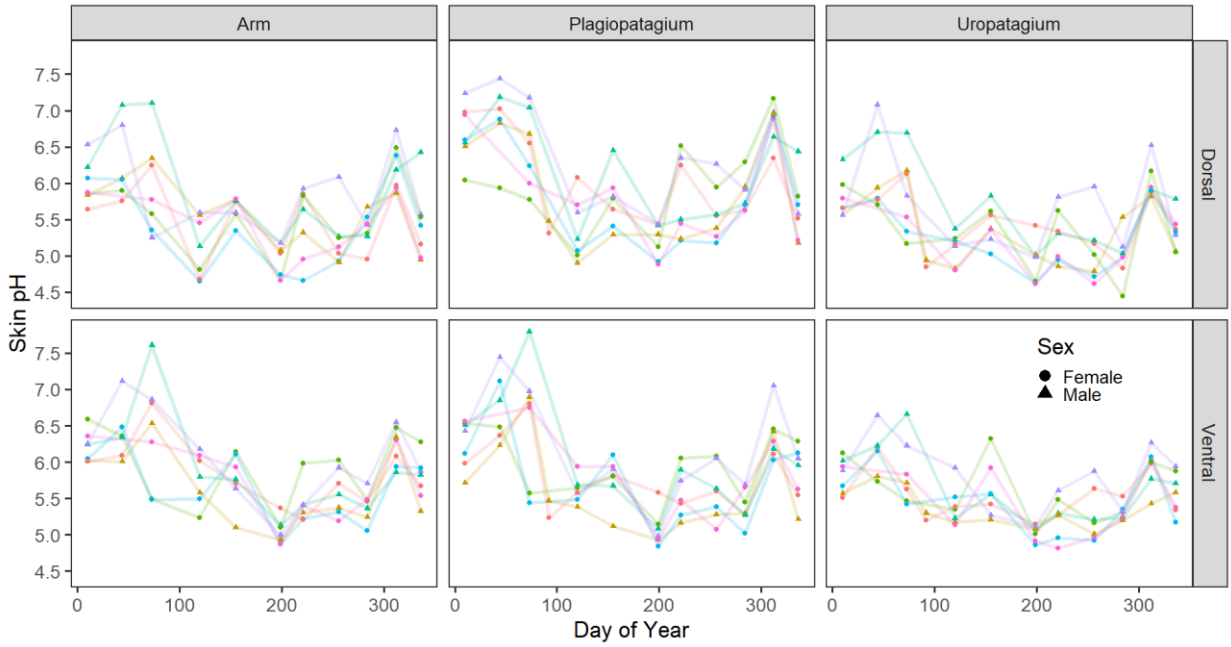

Figure 4.7**:** Skin pH from seven bats in the captive *Eptesicus fuscus* colony in Hamilton, Ontario that we measured 10 – 11 times each in 2019 – 2020. Each color indicates an individual bat. The availability of individuals in the colony varied over time; the individuals that were measured most frequently are depicted.

SUPPLEMENTARY MATERIAL CAPTIONS

Table 4.S1: Summary of previous research on the skin pH of non-human vertebrates. 'Months' indicates when measurements were taken. Whether the hair/fur of animals were shaved or clipped prior to measurement is indicated, although some animals and neonates are naturally hairless. When available, pH ranges are given in brackets in the 'mean pH' column. Articles on the skin pH of laboratory mice are representative of a larger literature body (Draize, 1942; Roy, 1954; Grono, 1970; Jenkinson and Mabon, 1973; Bartels *et al.*, 1991; Meyer and Neurand, 1991; Meyer *et al.*, 1991, 2001; Bogacz, 1992; Mauro *et al.*, 1998; Ruedisueli *et al.*, 1998; Ajito *et al.*, 2001; Fluhr *et al.*, 2001; Dunstan *et al.*, 2002; Hamann *et al.*, 2002; Behne *et al.*, 2002, 2003; Tsui *et al.*, 2002; Young *et al.*, 2002; Fox *et al.*, 2003; Hachem *et al.*, 2003, 2005, 2010; Matousek *et al.*, 2003; J. W. Fluhr *et al.*, 2004; J.W. Fluhr *et al.*, 2004; Popiel and Nicpoń, 2004; Bourdeau *et al.*, 2004; Zecconi *et al.*, 2005; Litwiller *et al.*, 2006; Choi *et al.*, 2007; Hatano *et al.*, 2009; Oh and Oh, 2009; Ferreira, 2010; Pan *et al.*, 2010; Szczepanik *et al.*, 2011, 2012, 2013; Breathnach *et al.*, 2011; Woodhams *et al.*, 2012; Moniaga *et al.*, 2013; Danciu *et al.*, 2014; Gołyński *et al.*, 2014; Lee *et al.*, 2014, 2016; Sakai *et al.*, 2014; Zajac *et al.*, 2015; Jang *et al.*, 2016; Bradley *et al.*, 2016; Hobi *et al.*, 2017; Koziol *et al.*, 2017; Mašínová *et al.*, 2017; Joly, 2018; Klinger *et al.*, 2018; Urnau, 2018; Cobiella *et al.*, 2019; Menon *et al.*, 2019; Proksch and Neumann, 2019; Barnhart *et al.*, 2020; Santoro *et al.*, 2021b, 2021a; Tang *et al.*, 2021; Wen *et al.*, 2021).

Table 4.S2: Sample sizes for each bat species in each month. The province where bats were measured is indicated after the sample size O=Ontario, N= New Brunswick, P=Prince Edward Island, Q=Quebec. Captive *Eptesicus fuscus* were sampled in Hamilton, Ontario. F = female and M = male.

Figure 4.S1: Daily maximum and minimum temperatures recorded April 2019 – March 2020 inside and outside the captive *Eptesicus fuscus* colony in Hamilton, Ontario. Markers represent individual data points while the lines indicate the mean with 95% confidence intervals in gray shading.

Figure 4.S2: Sites where wild bats were sampled over multiple time points. Sites that were sampled only twice and with two days or less between visits were excluded.

Figure 4.S3: Skin pH of seven wild bats in Ontario that were captured two to three times over summer 2019. Each color indicates an individual bat.

Figure 4.S4: Range in skin pH values among the six body parts measured on individual wild bats. Range was calculated by subtracting the lowest from the highest value on each bat.

FUNDING

This work was supported by New Brunswick Department of Energy & Resource Development; New Brunswick Wildlife Trust Fund to KJV and DFM [B309-145]; Trent University; Ontario Ministry of Northern Development, Mines, Natural Resources and Forestry); Sigma Xi to KJV [G201903158417906]; Discovery Grants awarded to C. M. D. and P.A.F. from the Natural Sciences and Engineering Research Council of Canada; and the New Brunswick Museum.

ACKNOWLEDGEMENTS

We are profoundly grateful to the many people who helped with field work: Yan Bourque, Lucy Brown, Lisa Browning, Mark Browning, Laura Bruce, David Christie, Maddie Empey, Brock Fenton, Janet Greenhorn, Adam Grottoli, Lesley Hale, Hannah Hamblin, Allie Hjort, Ryan Holt, Lauren Hooton, Howard Huynh, Sophia Konieczka, Jennifer Larkin, Tessa McBurney, Emily McNaughton, Kevin Quirion-Poirier, Carrie Sadowski, Laura Scott, Jordi Segers, Véronique St-

Hilaire, Andrew Sullivan, Olivier Trudel, Dylan White, and Val Von Zuben. We appreciate Erica Newton's help with Figure 4.2 and James Paterson's statistical advice. We also thank Dr. Kathleen Delanay, Dawn Graham, and the staff of the Psychology Animal Facility at McMaster University for veterinary and animal care of the captive bat colony. Access to hibernacula on private lands in New Brunswick and Quebec was generously provided by D. Roberts, J. Chown, Arbraska Laflèche, and Tony Gilchrist. Scientific permits for entering sites in New Brunswick were provided by the New Brunswick Department of Natural Resources and Energy Development Species-at-Risk Program and the New Brunswick Protected Natural Areas Program. Bats for the McMaster colony were captured under Wildlife Scientific Collector's Authorizations from the MNDNRF.

CHAPTER 5

PREFACE

Title: A review of sebum composition and function in wild mammals: skin diseases, disease resistance, and microbiomes

Authors: Karen J. Vanderwolf, Christopher J. Kyle, and Christina M. Davy.

Reference: submitted to *Physiological and Biochemical Zoology* Impact Factor: 2.291

Contributions: C.M.D., K.J.V., and C.J.K. conceived the research presented in this study. K.J.V. conducted the literature search, created Table 5.1 and Fig. 5.1, drafted the manuscript, and made revisions according to coauthor's recommendations. C.J.K. edited the initial draft. All authors edited the manuscript prior to submission. C.M.D. agreed that the contributions of K.J.V. are as stated, above.

CHAPTER 5

A review of sebum composition and function in wild mammals: skin diseases, disease resistance, and microbiomes

Submitted to *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*

ABSTRACT

Diseases vary among and within species but causes of this variation can be unclear. Immune responses are an important driver of disease variation, but mechanisms on how the body resists pathogen establishment before immune responses are activated are understudied. Skin surfaces of mammals are the first line of defense against abiotic stressors and pathogens, and skin attributes such as pH, microbiomes, and lipids influence disease outcomes. Sebaceous glands produce sebum that is composed of multiple types of lipids with species-specific compositions. Sebum affects skin barrier function by minimizing water loss, supporting thermoregulation, protecting against pathogens, and preventing UV-induced damage. Sebum affects skin microbiome composition, as sebum has antimicrobial properties, but is also a potential nutrient source. Intra- and interspecific variation in sebum composition influences skin disease outcomes in humans and domestic mammal species but is not well-characterized in wildlife. We synthesized knowledge on sebum function in wild mammals in relation to skin diseases and microbiomes. Current literature describes sebum composition of only 29 live, wild mammalian species. Sebum is important in dermatophilosis, various forms of dermatitis, demodicosis, and potentially white-nose syndrome that negatively impact wildlife populations.

Sebum composition likely affects disease susceptibility, as lipid components can have effective antimicrobial functions against specific pathogens. It is unclear why sebum composition is species-specific, but both phylogeny and environmental effects may drive differences. Our review summarizes sebum function and influence on skin microbes in context of skin diseases to inform future studies on this understudied topic and elucidate mechanisms of disease resistance.

INTRODUCTION

Understanding how some species or populations resist disease can inform management strategies, yet the underlying mechanisms leading to varied disease outcomes are poorly understood. Immune functions are an important driver of variation in responses to disease, but mechanisms on how the body resists pathogen entry and establishment before immune responses are activated are understudied. Pathogens can enter the body through mucosal surfaces in the gastrointestinal, urogenital, and respiratory tracts (van Ginkel *et al.*, 2000), as well as the skin. The skin surface of mammals is the main interface with the external environment, and the initial physical and chemical barrier to pathogens.

Skin is an effective barrier to the outside environment, nevertheless a variety of skin diseases occur in mammals, caused by bacteria, fungi, viruses, environmental stressors (UV damage, chemical exposure), and invertebrate parasites (Beckmen *et al.*, 1997; Munday *et al.*, 1998; Bressem *et al.*, 2009; Martinez-Levasseur *et al.*, 2011; Simpson *et al.*, 2013; Lorch *et al.*, 2015; Goodnight, 2015; Muneza *et al.*, 2016; Fountain *et al.*, 2017, 2019; Akdesir *et al.*, 2018; Chuma *et al.*, 2018; Doneley and Sprohnle-Barrera, 2021; Kiula *et al.*, 2021; Le Barzic *et al.*, 2021). Infection with one skin disease can compromise skin defenses and increase susceptibility to other diseases (Fitzgerald *et al.*, 2008). Some skin diseases, such as sarcoptic mange, can

result in significant population declines and localized extinctions (Pence and Ueckermann, 2002; Dagleish *et al.*, 2007; Cypher *et al.*, 2017; Cheng *et al.*, 2021; Escobar *et al.*, 2021). For example, white-nose syndrome is a fungal skin disease that has killed millions of bats of multiple species in the last 15 years, and some species are now listed as endangered in North America due to the effects of the disease (Cheng *et al.*, 2021). Dermatophilosis, a skin disease caused by bacteria, can cause major economic losses to livestock owners owing to the downgrading of skin/wool, lower meat and milk production, and mortality of stock (Zaria, 1993; Msami *et al.*, 2001; Ayalew *et al.*, 2015). Additionally, new skin diseases are being discovered, such as the recent appearance of a skin disease of unknown etiology in numerous giraffe (*Giraffa camelopardalis*) populations across Africa (Muneza *et al.*, 2016).

Skin diseases of wild mammals vary among and within species in terms of both occurrence and severity, but mechanisms influencing this variation are not fully understood (Pence and Ueckermann, 2002; Oleaga *et al.*, 2012; Nimmervoll *et al.*, 2013; Langwig *et al.*, 2016; Akdesir *et al.*, 2018; Escobar *et al.*, 2021; Ringwaldt *et al.*, 2021). Potential mechanisms include variation in host immune responses, pathogen lineage, host behavior, abiotic factors, skin microbiomes, and skin physiology (Nimmervoll *et al.*, 2013; Moore *et al.*, 2018; Davy *et al.*, 2020; Turchetto *et al.*, 2020; Vanderwolf *et al.*, 2021a). Clarifying the role of skin physiology in skin disease origin and progression may lead to more effective treatments. This is particularly relevant for captive wild mammals, such as breeding populations of endangered species in zoos (Conde *et al.*, 2011), that can develop chronic and sometimes lethal skin diseases despite the provision of treatment and supportive care (Nutting and Dailey, 1980; Montali *et al.*, 1981; Hubbard *et al.*, 1983; Dunn *et al.*, 1984; Diniz *et al.*, 1995; Bauwens *et al.*, 1996; Brack *et al.*, 1997; Munson *et al.*, 1998; Pollock *et al.*, 2000; James and Raphael, 2000; Takle *et al.*, 2010;

Muneza *et al.*, 2016; Fountain *et al.*, 2017; Kloft *et al.*, 2019). Some skin diseases are not influenced by skin attributes, as pathogens can elude the skin barrier by entering the body through mucosal surfaces, insect bites, or skin trauma, such as lumpy skin disease in wild and domestic bovines, Tasmanian devil facial tumour disease, and histoplasmosis in mustelids (Akdesir *et al.*, 2018; Cunningham *et al.*, 2021; Namazi and Tafti, 2021). Nevertheless, skin characteristics play an important role in susceptibility to a range of diseases.

Skin surface defense against microbial invasion includes the combined effects of lack of water, epidermal desquamation, acidic pH, nutrient limitations, commensal microbes, antimicrobial lipids, and antibodies (Harder et al. 2013; Naik et al. 2012). Physiological and morphological differences across the integument, such as presence of hair and glands, can cause variations in temperature, pH, moisture, nutrient availability, and the composition of antimicrobial peptides and lipids such as sebum (Kearney *et al.*, 1984; Grice *et al.*, 2009; Findley *et al.*, 2013; Schommer and Gallo, 2013). This variation effectively creates diverse microhabitats across the surface of the skin that affect the density and diversity of microbial colonization, including pathogens (Kearney *et al.*, 1984; Harder *et al.*, 2013). Skin microbiomes have been studied in a variety of wild and domestic mammals, but the mechanisms driving interspecific, inter-population, and inter-individual variation in microbiome composition have rarely been investigated (Ross *et al.*, 2019).

Sebum coats the epidermis and hair or fur and is a major potential nutrient source for microbes (Harder et al. 2013; Kearney et al. 1984; Naik et al. 2012). Sebum generally includes cell debris and nonpolar (neutral) lipids, namely triacylglycerol, diacylglycerol, wax esters, squalene, cholesterol, sterol esters, and free fatty acids (Smith and Thiboutot, 2008). Some of these lipids also occur in other tissues or cell types, but squalene and wax esters are unique to

sebum (Smith and Thiboutot, 2008; Pappas, 2009). Substantial microbial populations occur in sebaceous glands and associated hair follicles in humans and domestic mammals (Harder et al. 2013; Kearney et al. 1984; Naik et al. 2012). The microbial diversity of sebaceous-rich skin sites in humans, such as the face and upper body, differs in composition and quantity from dry sites such as the forearm and buttock (Sanmiguel and Grice, 2015). Mammalian skin lipid composition can also negatively affect microbial growth, attachment to skin, and the production of virulence factors (Drake *et al.*, 2008; Fischer *et al.*, 2014). Given these patterns in humans and domestic mammals, sebum quantity and composition likely also affect skin microbiome quantity and composition in wild mammals, but this has yet to be examined.

Functions of sebum include contributing toward the establishment of the skin barrier by minimizing water loss, supporting thermoregulation, protecting against pathogens, and preventing UV-induced damage (Zouboulis *et al.*, 2008; Desbois and Smith, 2010; Lovászi *et al.*, 2018). Most knowledge about sebum functions derives from human studies and to a lesser extent from studies on domestic and laboratory mammals. Previous reviews focused on sebum composition and biochemistry in mammalian species, but did not address skin diseases or sebum function (Nikkari, 1974; Stewart and Downing, 1991). The emergence of white-nose syndrome in bats has spurred new interest in the sebum composition of wild mammals and functions of skin lipids in cutaneous disease (Pannkuk *et al.*, 2015; Ingala *et al.*, 2017; Frank *et al.*, 2018).

Altered sebum composition and quantity are associated with human skin diseases such as sebaceous gland hyperplasia in acne and hypoplasia in atopic dermatitis (Zouboulis *et al.*, 2008; Shi *et al.*, 2015; Knox and O'Boyle, 2021), and may also play a role in wildlife diseases. Sebum composition is species-specific among characterized taxa, both in lipid concentration and type (Nicolaides *et al.*, 1968; Lindholm *et al.*, 1981; Stewart and Downing, 1991), and may partially

explain why diseases associated with sebaceous glands also differ between species. For example, acne commonly occurs in human sebaceous follicles, but is rarely observed in other mammalian species, potentially due to the unique composition of human sebum (Shannon, 2020). Skin lipids are thought to play an important role in resistance to the skin disease dermatophilosis in multiple mammalian taxa (Zaria, 1993). The antifungal properties of sebum and interspecific variation in sebum composition may partially explain interspecific differences in skin disease outcomes among bats (Pannkuk *et al.*, 2015; Ingala *et al.*, 2017; Frank *et al.*, 2018).

Our objective is to synthesize existing literature concerning sebum function in wild mammals as it relates to skin diseases and microbiomes to identify key knowledge gaps for future research. Sebum influences both skin microbiomes and skin disease progression in humans and domestic mammals (Sanmiguel and Grice, 2015; Knox and O'Boyle, 2021). These mechanisms may also apply to wild mammals, so we also draw on studies of sebum function in laboratory and domestic mammals. Knowledge of sebum function may lead to a greater understanding of emerging diseases and interspecific differences in susceptibility among wild mammals. We explore the following topics: 1) sebum function in mammals, 2) how sebum affects skin microbiomes, 3) skin diseases involving sebum, 4) factors influencing sebum composition and quantity among mammals, and 5) directions for future research on sebum in wild mammals.

METHODS: DATABASE SEARCH AND LITERATURE SCREENING

To identify relevant literature, we searched Web of Science and Google Scholar using the search string: [(sebum OR sebaceous) AND (composition OR composed OR function OR epidermis OR epidermal OR skin OR epidemiology OR disease OR fungi) AND (bat OR

wildlife OR mammal)]. The exclusion phrase [-human -children] was included in the Google Scholar search to exclude acne literature and to focus on wild mammals. We ordered the Google Scholar search results by relevance. We retained peer-reviewed publications that described sebum composition or sebum function in relation to skin diseases of any wild mammalian species. We excluded articles about scent marking/behavior, skin treatments in humans and domestic or laboratory animals, or histology (physical structure of skin, hair, and glands) to focus on sebum function in relation to skin disease (Figure 5.1). We summarized studies on the sebum composition of wild mammals in Table 5.1. We included a total of 287 articles in our final review, the results of which are described below.

SEBACEOUS GLAND OCCURRENCE AND FUNCTION

Sebaceous glands are absent in a number of species that are hairless or have a sparse distribution of fur or hair, including the Cetacea (whales, dolphins, porpoises), Hippopotamidae (hippos), Elephantidae (elephants), naked mole-rat (*Heterocephalus glaber*), and sirenians (Dugongidae, Trichechidae) (Daly and Buffenstein, 1998; Springer and Gatesy, 2018; Lopes-Marques *et al.*, 2019; Menon *et al.*, 2019; Springer *et al.*, 2021). In rhinoceros species sebaceous glands are either absent or poorly developed (Springer and Gatesy, 2018). In pangolins (*Manis* spp.) and desert hedgehogs (*Paraechinus aethiopicus*) sebaceous glands are restricted to the snout and abdomen (Springer and Gatesy, 2018; Massoud, 2020). Sebaceous glands are also absent in some species with fur, namely Cynocephalidae (colugos) (Springer and Gatesy, 2018), but further information on colugo's skin properties is unavailable. Aside from these exceptions, sebaceous glands are nearly ubiquitous, though unevenly distributed, in the hair-bearing skin of most mammals.

Sebaceous glands are composed of sebum-producing cells (sebocytes) that release their contents onto the skin surface via hair canals (Thody and Shuster, 1989; Zouboulis *et al.*, 2008). Sebocytes undergo a maturation process followed by a cell-type specific cell death that results in the holocrine secretion of sebum (Thody and Shuster, 1989; Zouboulis *et al.*, 2008). Sebaceous glands are usually found in association with hair follicles forming a pilosebaceous unit, with the sebaceous gland located in the upper portion of the hair follicle where it is not affected by the hair cycle (the four stages of growth and loss of hair) (Smith and Thiboutot, 2008). Some sebaceous glands occur without an associated hair follicle, such as the meibomian glands (eyelids), Fordyce's spots (oral epithelium), and the ceruminous glands (ears) (Smith and Thiboutot, 2008; Zouboulis *et al.*, 2016). Sebaceous gland volume is partially determined by the surface area of the hair follicle, although not for those associated with vibrissae (Haffner, 1998). The amount of sebum produced at a particular time is governed by the gland size and the number of secreting cells (Sokolov, 1982; Makrantonaki *et al.*, 2011). Ordinary sebaceous glands produce a continuous flow of sebum, resulting in constant lubrication of hair and skin (Sokolov, 1982). Changes in the composition of skin surface lipids have been used as an index of sebaceous gland activity. The palmitate-to-stearate and the stearate-to-oleate ratios are positively correlated with sebaceous gland secretion rate in the rat, and the rate of squalene synthesis may be positively correlated with gland size in humans (Nikkari and Valavaara, 1970; Strauss *et al.*, 1976; Thody and Shuster, 1989). Lipids are generated not just by sebaceous glands, but also within the epidermis by keratinocytes (Shi *et al.*, 2015). The composition of lipids produced by sebaceous glands and the epidermis differs despite some overlap (Stewart and Downing, 1991; Pappas, 2009; Butovich, 2017). Studies on the skin lipids of mammals do not always differentiate between epidermal and sebaceous lipids (Pappas, 2009), which complicates

interpretation of lipid composition and function from different sources. Studies on epidermal lipids are not included in this review.

Many mammalian species have scent glands composed of enlarged and modified sebaceous glands that produce chemical signals communicating information about species, sex, individual identity, reproductive condition, and social status (Zouboulis *et al.*, 2008). Scent glands can contain secretions from multiple sources, including sebaceous glands, apocrine (sweat) glands, urine, feces, and saliva, and often contain pheromones and other substances that are not present in non-specialized sebaceous glands over the rest of the skin (Jenkinson *et al.*, 1967; Gassett *et al.*, 1996; Khazanehdari *et al.*, 1996; Waterhouse *et al.*, 1996; Salamon and Davies, 1998; Kannan and Archunan, 1999; Salamon *et al.*, 1999; Osborn *et al.*, 2000; Buesching *et al.*, 2002; Dingzhen *et al.*, 2006; Muñoz-Romo *et al.*, 2012; Martín *et al.*, 2014; Sergiel *et al.*, 2017; Adams *et al.*, 2018; Faulkes *et al.*, 2019). Studies generally report the composition of scent glands without differentiating which compounds originate from which source, consequently functions performed by scent glands cannot be specifically attributed to sebum. Therefore, we did not list studies on the composition of scent glands in Table 5.1 to maintain the focus of this review on non-specialized sebaceous glands distributed over the body. However, much of the literature on sebaceous glands in wild mammals focuses on scent glands and patterns in these studies may provide insight into non-specialized sebaceous glands over the rest of the body. The composition of secretions from scent glands varies with reproductive status, social status, body condition, season, sex, diet, and age in a variety of wild mammals, and there can be overlap with the lipid composition from sebaceous glands, although often the composition is different (Jenkinson *et al.*, 1967; Nikkari, 1974; Volkman *et al.*, 1978; Sokolov *et al.*, 1980; Rasmussen, 1988; Thody and Shuster, 1989; Gassett *et al.*, 1996; Khazanehdari *et al.*, 1996;

Waterhouse *et al.*, 1996; Salamon and Davies, 1998; Burger *et al.*, 1999; Kannan and Archunan, 1999; Salamon *et al.*, 1999; Buesching *et al.*, 2002; Wood *et al.*, 2005; Zabaras *et al.*, 2005; Dingzhen *et al.*, 2006; Nassar *et al.*, 2008; Muñoz-Romo *et al.*, 2012; Martín *et al.*, 2014; Rossini and Ungerfeld, 2016; Sergiel *et al.*, 2017; Adams *et al.*, 2018; Faulkes *et al.*, 2019). Scent gland secretion composition does not vary by sex in all species (Woolhouse *et al.*, 1994; Burger *et al.*, 2020). Scent glands are often sexually dimorphic, reflecting differences in breeding activity and responses to sex steroid hormones, and may play a role in pathogen defense, thermoregulatory responses, and maintaining skin barrier function (Quay, 1970; Forman, 2005). Components of scent gland secretions may have insecticidal properties which could reduce ectoparasite loads (Muñoz-Romo *et al.*, 2012). Further studies on the function of scent glands in mammals beyond communication are needed, as they may play an important role in disease resistance.

FUNCTIONS OF SEBUM

The importance of sebum for skin health in humans has been questioned because the sebaceous glands of prepubescent children are largely inactive and because adult skin with no sebaceous activity, namely palms and soles, functions well (Kligman, 1963; Stewart and Downing, 1991). Sebum production in humans is high at birth, which can lead to acne, but sebaceous glands shrink during childhood until puberty (Shannon, 2020). Multiple forms of dermatitis disappear with the onset of puberty and accompanying increase in sebaceous gland activity (Rothman *et al.*, 1946; Shi *et al.*, 2015; Wertz, 2018). It is unknown how active sebaceous glands must be to minimize water loss, support thermoregulation, protect against

pathogens, and prevent UV-induced damage. Sufficient sebum may be produced by children to fulfill these functions (Stewart and Downing, 1991).

The question of the importance of sebaceous glands also arises for wild mammals given that sebaceous glands are absent in some lineages (Daly and Buffenstein, 1998; Springer and Gatesy, 2018; Lopes-Marques *et al.*, 2019; Menon *et al.*, 2019; Springer *et al.*, 2021). Aside from colugos, these species are all characterized by a sparse distribution or absence of hair and fur, as are the palms and soles of humans that also lack sebaceous glands. Sebum is important for maintaining hair and fur as discussed below. Yet, it is unclear how species lacking sebaceous glands replace functions, outside of fur maintenance, performed by sebaceous glands in other mammals. Potential strategies include regular wetting or immersion of the skin in water, secretions from other glands with UV-protection and antimicrobial properties, and increased rates of epidermal desquamation to prevent colonization by microorganisms, ectoparasites, and macrosymbionts (Brown *et al.*, 1983; Hicks *et al.*, 1985; Lillywhite and Stein, 1987; Fish and Hui, 1991; Eltringham, 1999; Saikawa *et al.*, 2004; Martinez-Levasseur *et al.*, 2011, 2013).

Changes in sebum composition or quantity can be a cause or consequence of disease and can also impact the functions sebum performs. For instance, compromised skin barrier function, which is assessed by measuring rates of cutaneous water loss, is an indicator of various skin disorders (Ohman and Vahlquist, 1994; Muñoz-Garcia *et al.*, 2012; Knox and O'Boyle, 2021). Understanding how sebum functions in healthy conditions may provide insight into disease mechanisms. Below we review functions performed by sebum that are important in maintaining healthy skin.

Skin Barrier Function

A major challenge of terrestrial wildlife is to minimize water loss, especially in dry environments. If mammalian skin is damaged or diseased, cutaneous water loss can increase by several orders of magnitude (Lillywhite, 2006). Dry skin is linked to various forms of dermatitis and in extreme cases excessive cutaneous water loss can lead to death from dehydration (Nishifuji and Yoon, 2013). Dry skin can crack and disrupt the skin barrier, which then provides entry points for microbes (Nishifuji and Yoon, 2013). Epidermal lipids, rather than sebaceous lipids, are thought to play the dominant role in minimizing cutaneous water loss (Lillywhite, 2006). Compared to sebaceous lipids, epidermal lipid composition is similar among mammalian species, possibly due to evolutionary conservation of a fundamental mechanism for water retention, although data on only a few species are available (Nicolaides *et al.*, 1968; Birkby *et al.*, 1982; Wertz *et al.*, 1983). Sebum can also contribute to waterproofing the skin. Among armadillo species the greater development of sebaceous glands in Euphractinae compared to Dasypodinae is thought to prevent desiccation of cornified scales in extremely arid climates (Krmpotic *et al.*, 2015). Laboratory mice genetically engineered to have defective or missing sebaceous glands have disrupted hair cycles, dry hair, skin lesions, defective water repulsion, compromised thermoregulation, and chronic, progressive alopecia (hair loss) (Wood *et al.*, 2005; Zhang *et al.*, 2014). Laboratory mice with intact sebaceous glands mutated to lack various enzymes and proteins important for lipid metabolism and secretion on the skin surface developed atrophic sebaceous glands, defective production of skin lipids, and altered lipid composition accompanied by dry/brittle fur and hair loss (Chen *et al.*, 2002; Westerberg *et al.*, 2004; Zhang *et al.*, 2014). These mice also exhibited impaired water repulsion, increased rate of transepidermal water loss, and hypothermia after water immersion (Chen *et al.*, 2002; Westerberg *et al.*, 2004;

296 Zhang *et al.*, 2014). Sebaceous gland degeneration is characteristic of some types of alopecia in
297 humans and laboratory mice (Smith and Thiboutot, 2008; Pappas, 2009; Schneider and
298 Zouboulis, 2018). In *asebia* mutated mice, sebaceous gland hypoplasia is induced through
299 spontaneous mutation of the gene *ab* (Schneider, 2015). This mutation impairs production of
300 glycerol, a contributor of stratum corneum hydration, which emphasizes the importance of
301 glycerol generation from triglycerides in the sebaceous glands (Fluhr *et al.*, 2003). These
302 findings illustrate the importance of sebum in maintaining healthy skin and hair as part of host's
303 defenses against disease.

304 Sebum also plays a role in thermoregulation. An inability to thermoregulate can lead to
305 hypothermia or hyperthermia, both of which can result in death (Cheshire, 2016). At higher
306 temperatures, sebum acts as a surfactant for eccrine secretions in humans to retain sweat and
307 promote heat loss, as sweat that immediately drips off the skin does not effectively dissipate heat
308 (Nicolaides *et al.*, 1968; Porter, 2001). At lower temperatures, in its viscous form, sebum acts as
309 a local repellent of rain on exposed skin (Butcher and Coonin, 1949). Therefore, the outcome of
310 secretory interactions is for an external fluid, rain, to be projected off the skin in cool wet
311 conditions, whereas in hot conditions, the internally generated fluid, eccrine sweat, is encouraged
312 to spread in a film across the skin and be retained on the surface (Butcher and Coonin, 1949;
313 Nicolaides *et al.*, 1968; Porter, 2001). It is unclear if this is an important thermoregulatory
314 mechanism in hot conditions in other mammals since sweating is best known in humans
315 (eccrine) and horses (apocrine), although sweating also occurs in other mammals to a lesser
316 degree (Robertshaw, 1985). Sebum may play an unrecognized role in heat dissipation in multiple
317 mammalian taxa. Similar to humans, sebum creates a water-repellent pelage in wild mammals by
318 coating hair and fur to prevent over-wetting and resulting hypothermia (Waldorf and Vedros,

1978; Walro and Svendsen, 1982; Thody and Shuster, 1989; Porter, 2001; Zhang *et al.*, 2015). Effectively repelling water off the skin is not just important for thermoregulation. Excessive wetting softens the skin and disrupts normal cutaneous microflora, which can increase disease susceptibility (Tellam *et al.*, 2021).

While sebum-coated fur is important to repel water, excess lipids can cause the fur to mat, thus compromising insulative qualities (Harriman and Thiessen, 1983). Some species of rodents, such as kangaroo rats (*Dipodomys* spp.) and gerbils (*Meriones* spp.), groom and sandbathe to remove excess lipids from their fur (Randall, 1981; Thiessen and Pendergrass, 1985). Captive Mongolian gerbils (*Meriones unguiculatus*) living at 10ºC had significantly higher levels of pelage lipids than at 24ºC, suggesting a role of lipids in thermoregulation (Thiessen and Pendergrass, 1985). Individuals can alter pelage lipid quantity by either removing lipids through sandbathing or increasing lipids by autogrooming secretions from Harderian glands (Thiessen and Pendergrass, 1985). Harderian glands are present in a variety of mammals and are located near the eyes (Sakai, 1981). Removing Harderian glands, or shampooing animals, decreases the quantity of pelage lipids and decreases the ability of individuals to thermoregulate in cold environments, while increasing evaporative water loss in hot environments in both gerbils and muskrats (*Ondotra zibethicus*) (Thiessen and Kittrell, 1980; Harlow, 1984; Thiessen and Pendergrass, 1985). Thermoregulation is restored and evaporative water loss decreased by applying lipids or mineral oil to the skin (Thiessen and Kittrell, 1980; Harlow, 1984). This mechanism may also exist in other mammals, such as blind mole rats (*Spalax ehrenbergi*) (Shanas and Terkel, 1996).

Except for marsupials (Ferner, 2021), and species lacking sebaceous glands, fetal sebaceous glands activate during gestation and in humans they produce vernix caseosa, a white

lipid-rich biofilm covering the skin, in the last trimester of pregnancy (Shannon, 2020). Vernix contains both sebaceous lipids and epidermal lipids produced by the fetus (Hoath *et al.*, 2006; Nishijima *et al.*, 2019). The biological function of vernix caseosa is not well understood but is thought to be a barrier to water loss, assist thermoregulation after birth, have antimicrobial and anti-oxidant functions, facilitate skin surface acidification, and potentially act as a film to minimize friction during delivery (Visscher *et al.*, 2005; Hoath *et al.*, 2006; Wang *et al.*, 2018; Nishijima *et al.*, 2019; Shannon, 2020). The vernix lipid composition of California sea lions (*Zalophus californianus*), the only other mammal aside from humans known to produce vernix, is similar to human vernix (Wang *et al.*, 2018).

Protection Against Abiotic Stressors

A major challenge for terrestrial wildlife is protecting skin against oxidative stressors such as ultraviolet radiation, ozone, and chemicals. Oxidative stress regulates major signaling pathways of extrinsic skin aging and skin diseases like acne, various forms of dermatitis, and skin carcinogenesis (Briganti and Picardo, 2003; Masaki, 2010; Zouboulis *et al.*, 2016). Skin that is damaged by these stressors is more prone to infection because barrier function can be compromised (Zouboulis *et al.*, 2016). Sebum provides photoprotection but exposure to UV radiation can lead to cellular damage by changing the composition of skin lipids, such as increasing the percentage of free fatty acids and cholesterol, in humans and laboratory rodents (Gloor and Karenfeld, 1977; Ohsawa *et al.*, 1984; Picardo *et al.*, 1991; Marques *et al.*, 2002; Akitomo *et al.*, 2003; Mudiyanselage *et al.*, 2003; Zouboulis *et al.*, 2016). Exposure to UV radiation can also increase the amount of skin surface lipids from both epidermal lipids and sebaceous glands, depending on the dosage (Gloor and Karenfeld, 1977; Akitomo *et al.*, 2003).

Exposure to environmental pollutants and toxins can change skin lipid composition and inhibit lipogenesis in human sebaceous glands, and sebum is one of the skin's defenses against such toxins (Zouboulis *et al.*, 2016). Human sebaceous glands secrete vitamin E onto the upper layers of the skin which is protective again oxidation (Thiele *et al.*, 1999), but this has not been studied in wildlife. Disruptions of sebum's photoprotective properties can result in skin disorders. For example, skin lesions caused by UV-radiation are well documented in whales (Martinez-Levasseur *et al.*, 2011, 2013), a group of mammals that lack sebaceous glands (Springer *et al.*, 2021), although such lesions are not well known in other species that lack sebaceous glands.

Microbes and Sebum

Mammalian skin lipid composition can affect microbial growth, attachment to skin, and the production of virulence factors, but skin lipids can also be an important nutrient source for both commensal and pathogenic microbes (Drake *et al.*, 2008; Fischer *et al.*, 2014). The prevalence and biodiversity of microbes on skin is correlated with sebum quantity and skin hydration levels in humans (Mukherjee *et al.*, 2016). In humans the stimulation of sebaceous gland secretion by hormones at puberty favors lipophilic taxa on the skin, such as *Corynebacterium* spp. and *Propionibacterium* spp. which are considered normal components of skin microbiomes (Mukherjee *et al.*, 2016; Roux *et al.*, 2021). Skin microbiomes play a role in host defense against pathogens (Chen *et al.*, 2018). These finding show that sebum quantity and composition influence skin microbiomes, but microbiomes can also alter sebum composition. Bacteria secrete lipases which break down triglycerides secreted from sebaceous glands (Zouboulis, 2004; Drake *et al.*, 2008). Bacteria can also alter the composition of scent gland secretions in multiple wild mammal species through fermentation and breaking down proteins

and carbohydrates (Albone *et al.*, 1974; Studier and Lavoie, 1984; Woolhouse *et al.*, 1994; Burger *et al.*, 1999; Osborn *et al.*, 2000; Voigt *et al.*, 2005; Theis *et al.*, 2013; Gonzalez-Quinonez *et al.*, 2014). Impaired production or alteration of sebum composition have been proposed as key features in atopic dermatitis and susceptibility to microbial colonization (Zouboulis, 2004; Fischer *et al.*, 2014; Knox and O'Boyle, 2021). Components of human and laboratory mouse sebum, particularly fatty acids like lauric acid, oleic acid, sapienic acid, and palmitoleic acid, reduce growth of various pathogenic gram-positive bacteria, block adhesion to skin by fungi, and prevent germination of various dermatophytes (Bibel *et al.*, 1992; Wille and Kydonieus, 2003; Georgel *et al.*, 2005; Drake *et al.*, 2008; Chen *et al.*, 2011; Fischer *et al.*, 2014). For instance, skin deficient in free fatty acids is more susceptible to colonization by the opportunistic pathogen, *Staphylococcus aureus*, and protection against colonization is bolstered with the application of topical fatty acids (Georgel *et al.*, 2005; Takigawa *et al.*, 2005). Free fatty acids are produced via hydrolysis of their precursors, triglycerides secreted from sebaceous glands, by lipases secreted from commensal bacteria such as *P. acnes* and *Staphylococcus epidermidis* and by acid lipase produced by the epidermis (Zouboulis, 2004; Drake *et al.*, 2008). These findings illustrate that skin lipid composition influences skin microbiome composition and function, as well as disease susceptibility.

Free fatty acids may provide direct antibacterial activities against skin bacteria like *P. acnes*, and enhance the skin's innate antibacterial defense by inducing the expression of human β-defensin-2, an antimicrobial peptide, in human sebocytes and mouse skin (Nakatsuji *et al.*, 2010). Antimicrobial peptides and lipids on the skin can act synergistically against bacteria and yeast (Robertson *et al.*, 2006; Fischer *et al.*, 2014). Free fatty acids inhibit bacterial growth or induce death by cell lysis, inhibition of enzyme activity, impairment of nutrient uptake, and the

generation of toxic peroxidation and autooxidation products (Desbois and Smith, 2010). However, some skin pathogens, such as *Staphylococcus aureus*, are able to detoxify specific skin antimicrobial fatty acids (Subramanian *et al.*, 2019). Besides inhibiting or killing bacteria directly, free fatty acids also make conditions unfavorable for the growth of certain bacteria on the skin surface by maintaining an acidic pH (Fluhr *et al.*, 2001; Takigawa *et al.*, 2005). The antimicrobial activity of skin lipids varies with pH *in vitro*, with almost no activity >8pH (Bibel *et al.*, 1992). Sebaceous glands can synthesize and secrete either pro- and anti-inflammatory cytokines and lipids in response to environmental stimuli, such as the presence of microbes (Zouboulis, 2004; Zouboulis *et al.*, 2008; Lovászi *et al.*, 2018).

Most information available on the antimicrobial properties of wild mammal sebum derives from bats in North America. Recent research on the skin lipids of bats was prompted by the discovery of the fungus *Pseudogymnoascus destructans* (*Pd*) that causes white-nose syndrome (Lorch *et al.*, 2011a). The *Pd* hyphae can penetrate both the epidermis and dermis, causing severe skin lesions and destroying hair follicles, sebaceous glands, and sweat glands (Meteyer *et al.*, 2009, 2022; Courtin *et al.*, 2010). Research on the role of sebum in white-nose syndrome has focused on the antimicrobial properties of sebum against *Pd in vitro* rather than the disease itself. Sebum composition varies among bat species (Pannkuk *et al.*, 2012; Frank *et al.*, 2016) and changes in both composition and quantity over the hibernation season, both of which may affect *Pd* growth (Frank *et al.*, 2016, 2018; Ingala *et al.*, 2017). Infection with *Pd* changes the lipid composition of wing tissue (Pannkuk *et al.*, 2015). Some skin lipids of little brown myotis (*Myotis lucifugus*) and big brown bats (*Eptesicus fuscus*), such as 1-monopalmitolein, palmitoleic acid, pentadecanoic acid, and behenyl palmitoleate (wax ester), inhibit *Pd* growth *in vitro* (Ingala *et al.*, 2017; Frank *et al.*, 2018), but results differ depending on

incubation temperature and media type (Frank *et al.*, 2016; Ingala *et al.*, 2017; Gabriel *et al.*, 2019). The ability of some bats species, such as *E. fuscus*, to resist or tolerate *Pd* infection may be partially due to the wax ester, free fatty acid, and 1-monoacylglycerol composition of their skin lipids (Frank *et al.*, 2016, 2018). The epidermis of *E. fuscus* contains almost twice as much myristic, palmitoleic, and oleic acids as *M. lucifugus*, a white-nose syndrome-susceptible bat species, and these compounds all inhibit *Pd* growth *in vitro* (Frank *et al.*, 2016). Sebum from *M. myotis*, a European bat species that is highly resistant to cutaneous *Pd* infections, contains over 120 distinct types of wax esters (Řezanka *et al.*, 2015), some of which inhibit *Pd* growth *in vitro* (Frank *et al.*, 2018). Although *Pd* is not considered lipophilic, the fungus releases lipases, esterases, proteinases, and phospholipases that hydrolyze triacylglycerols, waxes, cholesterol esters, and glycerophospholipids to produce free fatty acids (Reynolds and Barton, 2014; Meteyer *et al.*, 2022). Hyphae are consistently seen at the openings of hair follicles and within sebaceous glands in infected bats (Meteyer *et al.*, 2022). Aside from bats, information on the antimicrobial properties of wild mammal sebum is available for only two other species. The free fatty acid portion of lipids that coat porcupine (*Erethizon dorsatum*) quills inhibits some bacteria strains *in vitro* (Roze *et al.*, 1990). Some fatty acids from northern fur seal (*Callorhinus ursinus*) skin, such as oleic acid and stearic acid, inhibited growth of five dermatophyte species *in vitro* (Waldorf and Vedros, 1978). As illustrated by white-nose syndrome, the antimicrobial properties of sebum are likely important in multiple skin diseases of wild mammals.

NON-HUMAN, MAMMALIAN SKIN DISEASES ASSOCIATED WITH SEBACEOUS GLANDS

Below we review all known skin diseases associated with sebum and sebaceous glands in wild mammals, apart from white-nose syndrome. We discussed the role of sebum in white-nose syndrome under the 'microbes and sebum' section above. We highlight potential functions of sebum in prevention or exacerbation of disease, and various consequences that can occur when normal functions performed by sebum are disrupted.

Dermatophilus congolensis and Dermatophilosis

The actinomycete bacterium *Dermatophilus congolensis* causes the skin disease dermatophilosis that presents as skin lesions characterized by an exudative dermatitis (Zaria, 1993; Ayalew *et al.*, 2015). *Dermatophilus congolensis* is not highly invasive and does not normally breach the barriers of healthy skin (Zaria, 1993; Ayalew *et al.*, 2015). It is considered a normal component of cutaneous microflora and likely requires a compromised skin barrier, such as minor wounds or transmission via insect bites, as a precursor to active infection (Zaria, 1993). During infection *D. congolensis* invades the keratinized layer of the skin along with hair follicles and sebaceous glands (Roberts, 1967). *Dermatophilus congolensis* secretes proteins, especially proteases to aid removal of the protective outer keratin layer of skin, lipases to remove skin lipids, and haemolysins to allow bacterial invasion of cells, that collectively facilitate invasion of the skin (How *et al.*, 1990). Infection rates are higher in young animals, potentially because skin barrier function is compromised since the skin lipid layer is not yet properly formed (Roberts, 1963a).

Dermatophilosis affects a wide range of domestic and wild mammalian species, including ungulates, rodents, bears, mustelids, monkeys, primates, and pinnipeds, although most knowledge of the disease derives from research on domestic sheep (Montali *et al.*, 1981; Salkin

and Gordon, 1983; Zaria, 1993; Brack *et al.*, 1997; Nemeth *et al.*, 2014; Ayalew *et al.*, 2015; Caron *et al.*, 2018). It can cause major economic losses to livestock owners, owing to the downgrading of skin/wool, lower meat and milk production, and mortality of stock (Zaria, 1993; Msami *et al.*, 2001; Ayalew *et al.*, 2015). Case fatality rates for dermatophilosis vary from 10-50% in some domestic species (Gitao *et al.*, 1998; Ayalew *et al.*, 2015). Mortality rates and non-lethal effects have not been quantified in wild mammals (Zaria, 1993). The effect of dermatophilosis on wild mammal populations may resemble domestic mammals, or it may differ due to lower host densities or other skin properties.

Increased rain and humidity leading to persistent wetting of the hair and skin are key environmental factors associated with *D. congolensis* infection (Zaria, 1993; Tellam *et al.*, 2021). The disease has a worldwide distribution but is most prevalent in humid tropical and subtropical regions, with mortality peaking during the rainy season (Zaria, 1993; Ayalew *et al.*, 2015). Lesion distribution in some species is concentrated in body regions such as the back that are prone to direct rain exposure (Le Riche, 1968; Dalis *et al.*, 2009). Prolonged exposure to moisture can disperse the protective lipid layer on the skin, change lipid composition, softens the skin, and disrupts normal cutaneous microflora, thereby increasing skin vulnerability to *D. congolensis* infection in sheep (Hay and Mills, 1982; James *et al.*, 1984; Colditz *et al.*, 2021; Tellam *et al.*, 2021). Moisture also promotes *D. congolensis* infection by causing the release of infective zoospores from infected scabs (Roberts, 1963b).

The mechanical properties of the sebaceous film as a barrier to bacteria and water appear to be more important in resisting infection than sebum's bacteriostatic action (Roberts, 1963a). Experimental infection of domestic sheep with *D. congolensis* without removing the sebaceous film produces only scattered lesions (Roberts, 1963a). Studies that experimentally challenge skin

with *D. congolensis* generally remove skin lipids before the addition of spores (Roberts, 1963a; Le Riche, 1968; Tellam *et al.*, 2021). Aside from antibiotics and vaccines, a topical treatment, Lamstreptocide, for the disease consists of sebaceous fatty acids such as palmitic, stearic, oleic, and linoleic acid (Zaria, 1993; Ayalew *et al.*, 2015). These results illustrate the protective properties of sebum against pathogens. *Dermatophilus congolensis* may also be inhibited by commensal microbes on the skin (Kingali *et al.*, 1990; Zaria, 1993).

Malassezia spp. and Dermatitis

The genus *Malassezia* consists of 18 species of dimorphic lipophilic yeasts that are common components of mammalian skin microbiomes (Batra *et al.*, 2005; Guillot and Bond, 2020). They are considered opportunistic skin pathogens, although causal relationships of *Malassezia* species with dermatological disorders are sometimes unclear (Batra *et al.*, 2005; Guillot and Bond, 2020). The genus is associated with skin conditions in humans such as dandruff, seborrheic dermatitis, atopic dermatitis, *Malassezia* folliculitis, psoriasis, and pityriasis versicolor (Gueho *et al.*, 1998; Ashbee and Evans, 2002; DeAngelis *et al.*, 2005; Harada *et al.*, 2015; Theelen *et al.*, 2018). Skin conditions associated with *Malassezia* often improve with anti-fungal treatment, which supports causal relationships of *Malassezia* with these skin disorders (Plant *et al.*, 1992; Harada *et al.*, 2015). *Malassezia* dermatitis and otitis is common in dogs but also found in other domesticated animals such as cats, pigs, cattle, horses, and goats (Batra *et al.*, 2005; Guillot and Bond, 2020).

Malassezia species cannot produce fatty acids themselves and require lipids from the environment for growth (Theelen *et al.*, 2018). *Malassezia* releases lipases, phospholipases, aspartyl proteases, and acid sphingomyelinases that hydrolyze lipid sources like sebum

triglycerides to obtain fatty acids (Ashbee and Evans, 2002; Celis *et al.*, 2017). These enzymes enable growth of these yeasts on host skin and change host sebum composition (Celis *et al.*, 2017). The *Malassezia* lipases are non-specific and degrade any available triglycerides (Ro and Dawson, 2005). The saturated fatty acids are consumed, and the abundant unsaturated fatty acids are left on the skin (Guého *et al.*, 1996; Ro and Dawson, 2005). The unsaturated free fatty acids hydrolyzed from triglycerides by *Malassezia*, such as oleic acid and arachidonic acid, can result in inflammation, irritation, scaling, and skin barrier defects in susceptible individuals (Ashbee and Evans, 2002; Gupta *et al.*, 2004; DeAngelis *et al.*, 2005; Ro and Dawson, 2005; Harada *et al.*, 2015). Indeed, applying oleic acid to human scalps can induce flaking in dandruff-susceptible but not non-susceptible individuals (DeAngelis *et al.*, 2005). *Malassezia* interact with their host directly via chemical mediators and indirectly through immune interplay, so both host immunity and host barrier function have roles in *Malassezia*-associated skin disorders (Wikramanayake *et al.*, 2019).

Since sebum is an important nutrient source for *Malassezia*, diseases that cause increased sebum production, such as some endocrine and bacterial skin diseases, provide a cutaneous microenvironment that encourages overgrowth of *Malassezia* spp. (Batra *et al.*, 2005). Although *Malassezia* can be found on skin poor in sebum, such as the toe web space and palms, it is most abundant on body parts rich in sebum such as the face and scalp that are also the most common areas for skin disorders associated with *Malassezia*, such as seborrheic dermatitis and pityriasis versicolor (Gueho *et al.*, 1998; Findley *et al.*, 2013; Harada *et al.*, 2015; Jo *et al.*, 2017). In humans, age and sex are associated with changes in *Malassezia* composition on the skin as well as *Malassezia*-associated skin disorders, likely due to differences in the activity of sebaceous glands driven by hormones (Ashbee and Evans, 2002; Ro and Dawson, 2005). Other

disturbances of skin microenvironmental factors, such as temperature, humidity, and skin pH, can also contribute to the development of dermatomycosis (Hađina *et al.*, 2019).

Research on *Malassezia* in wildlife has documented the genus on the skin of various wild mammals but has not explored its association with skin disorders. It has been isolated from free-ranging species with sarcoptic mange such as red fox (*Vulpes fulva*), porcupine (*Erethizon dorsatum*), and coyote (*Canis latrans*), and also from zoo animals with dermatitis such as Indian rhinoceros (*Rhinoceros unicornis*), white rhinoceros (*Ceratotherium simum simum*), South American sea lions (*Otaria byronia*), gray seal (*Halichoerus grypus*), harbor seal (*Phoca vitulina*), and California sea lions (*Zalophus californianus*) (Weidman, 1925; Salkin *et al.*, 1980; Bauwens *et al.*, 1996; Guillot *et al.*, 1998; Nakagaki *et al.*, 2000; Pollock *et al.*, 2000; Nimmervoll *et al.*, 2013; Hađina *et al.*, 2019). However, *Malassezia* spp. are also present on a variety of free-ranging and captive mammal species with no skin disease (Kuttin and Müller, 1994; Wesche and Bond, 2003; Dall' Acqua Coutinho *et al.*, 2006; Gandra *et al.*, 2008; Neves *et al.*, 2017; Lorch *et al.*, 2018; Coutinho *et al.*, 2020). Given its occurrence in domestic mammals, *Malassezia* dermatitis and otitis likely also occurs in wild mammals.

Propionibacterium acnes **and acne**

Acne is primarily a human disease, although minor forms of acne occur in dogs and cats (Shannon, 2020). This may be partially due to the differences in sebum composition among species. For example, sapienic acid is a sebaceous fatty acid unique to humans and is implicated in the development of acne (Shannon, 2020). Sebum composition on skin with acne differs from unaffected skin, as patients produce sebum with more squalene and decreased levels of linoleic acid (Pappas *et al.*, 2009; Melnik, 2015; Shi *et al.*, 2015; Knox and O'Boyle, 2021). The

pathogenesis of acne includes increased production of sebum (as occurs during adolescence in humans), blockage of the pilosebaceous unit, increased inflammation, and increased quantity of bacteria (Zouboulis, 2004; Shi *et al.*, 2015; Suh and Kwon, 2015). Acne in dogs and cats primarily occurs on the chin, but the pathogenesis is largely unknown (Plewig and Kligman, 2000).

The bacterium *Propionibacterium acnes* is associated with acne and is more prevalent on sebaceous body parts where sebum is its nutrient source (Smith and Thiboutot, 2008; Shi *et al.*, 2015). Lipases and peroxidases produced by the bacteria cleave sebaceous triglycerides into glycerol and free fatty acids, such as palmitic acid, which are inflammatory, as well as oxidizing squalene (Melnik, 2015). Increases in palmitic and oleic acid on the skin are thought to drive comedogenesis and further microbial colonization of the skin (Melnik, 2015; Lovászi *et al.*, 2018). Sebum composition affects *P. acnes* adherence and growth on the skin (Melnik, 2015). *Propionibacterium acnes* is not common on domestic animals, possibly due to sebum composition, but has been found on guinea pigs, cats, and dogs (Webster *et al.*, 1981). The only report from wild mammals we are aware of is *Propionibacterium* sp. on a beaver (*Castor canadensis*) (Rogovskyy *et al.*, 2012).

Ectoparasites and Demodicosis/Dermatitis

A variety of ectoparasites, such as lice (*Trichodectes* spp.), feed on sebaceous secretions and can cause skin problems (Jimenez *et al.*, 2010), but hair follicle mites (*Demodex* spp.), are specialized to live in sebaceous glands (Izdebska, 2009). *Demodex* spp. parasitize a wide range of domesticated and wild mammalian species (Sastre *et al.*, 2016; Jańczak *et al.*, 2017). Mites occupy the sebaceous gland portion of the pilosebaceous complex and feed on sebum and

epithelia, generally without causing any clinical signs such as inflammation or lesions (Mauldin and Peters-Kennedy, 2015; Jańczak *et al.*, 2017). The greatest concentration of mites occurs in areas of the body rich in sebaceous glands (Jimenez-Acosta *et al.*, 1989; Mauldin and Peters-Kennedy, 2015). Mites can become pathogenic when they proliferate excessively in response to changes in the host's cutaneous environment or immune response, leading to skin conditions such as demodicosis (demodectic or red mange), seborrheic dermatitis, and potentially rosacea (Sastre *et al.*, 2016; Jańczak *et al.*, 2017; Forton and Maertelaer, 2021). Demodicosis can result in severe alopecia (De Bosschere *et al.*, 2007; Barlow and Wood, 2011). Demodicosis is well known in humans, cats, and dogs but is generally considered rare in other domestic species, although local outbreaks occur (Nutting *et al.*, 1975; Mauldin and Peters-Kennedy, 2015). *Demodex* mites contain lipase enzymes and the hydrolysis of sebum triglycerides releases fatty acids with irritant properties (Jimenez-Acosta *et al.*, 1989). Human patients with demodicosis have altered sebum composition, although it is unclear if this is a cause or consequence of the disease (Demİrdağ *et al.*, 2016). Demodicosis has been reported in a variety of captive and free-ranging wild mammals (Carpenter *et al.*, 1972; Nutting and Dailey, 1980; Dräger and Paine, 1980; Pence *et al.*, 1981; Forrester *et al.*, 1993; James and Raphael, 2000; De Bosschere *et al.*, 2007; Gentes *et al.*, 2007; Wolhuter *et al.*, 2009; Takle *et al.*, 2010; Barlow and Wood, 2011; Nemeth *et al.*, 2014; Salvadori *et al.*, 2016; Sastre *et al.*, 2016; Bianco *et al.*, 2019; Javeed *et al.*, 2021).

Ticks and mites, such as *Dermacentor* spp. and *Sarcoptes* spp., are attracted to specific components of skin lipids which may partially explain differences in occurrence among host species and body parts (Arlian and Vyszenski-Moher, 1995). Variations in the composition of skin secretions may also play a role in the attractiveness of hosts to tsetse flies (Gikonyo *et al.*,

2002). Sebaceous gland hyperplasia and seborrhoea (excessively oily skin) are some of the symptoms of sarcoptic mange caused by the mite *Sarcoptes scabiei* (Bornstein *et al.*, 1995; Oleaga *et al.*, 2012).

Cancer

Skin tumors can develop in sebaceous glands, and are sometimes associated with papillomaviruses (Sundberg *et al.*, 1988; Casanova *et al.*, 2017). Sebaceous gland adenoma and carcinoma have been documented in a variety of captive wild mammals, but only once in a free-ranging individual (Baird's Tapir, *Tapirus bairdii*) (Hubbard *et al.*, 1983; Sundberg *et al.*, 1988; Canfield *et al.*, 1990; Obendorf, 1993; Owston *et al.*, 2008; Bharathidasan *et al.*, 2014; Majie *et al.*, 2014; Matute *et al.*, 2014; Arguedas *et al.*, 2019; Kloft *et al.*, 2019). The prevalence, pathogenesis, and population effects of these tumors in wild mammals is unknown. It is also unclear whether sebum plays a role in cancer development.

FACTORS AFFECTING SEBUM COMPOSITION AND QUANTITY AMONG MAMMALS

Lipid classes found in mammal sebum include sterol esters, wax esters, squalene, hydroxyacid diesters, diol diesters, ω-lactones, glyceryl ethers, wax triesters, triglycerides, sterol diesters, free fatty acids, free sterols, free fatty alcohols, and unidentified compounds (Stewart and Downing, 1991). However, the sebum composition of only 29 live, wild mammalian species have been described so additional compounds may occur in uncharacterized species (Table 5.1). Bats are now the best-studied taxa with regards to sebum composition among wild mammals due to interest generated by the skin disease white-nose syndrome. Lipids from the fur of dead

mammals (roadkill and skins in collections) were characterized for a further 22 wild mammalian species (Lindholm *et al.*, 1981), but sebum may change after death, decomposition, or taxidermic preparation. The reported composition of skin lipids varies depending on the selected method of sampling and analysis (Pappas, 2009). This complicates comparisons among studies as different methods were used to target different classes of lipids.

Each mammal species characterized to date produces sebum of a unique composition (Lindholm *et al.*, 1981; Stewart and Downing, 1991). There are similarities within some families and genera, such as within Canidae (similarities in diesters and cholesteryl esters) and among *Equus* spp. (similarities in wax diesters, lactones, and cholesteryl esters) (Lindholm *et al.*, 1981; Stewart and Downing, 1991). However, lipid composition can also be quite different within families, such as Sciuridae (differences in the presence of triolein and stearyl oleate) and Mustelidae (differences in the presence of triolein, stearyl oleate, cholesteryl oleate, and squalene) (Lindholm *et al.*, 1981). There are large differences in sebum composition among families as different classes of lipids are present (Lindholm *et al.*, 1981). These observations indicate that phylogeny partially explains some patterns in sebum composition but does not fully account for inter-species variation.

Ecological associations may be another factor influencing sebum composition. Several authors have noted that some aquatic or semi-aquatic mammals have large amounts of squalene, such as sea otters (*Enhydra lutris*), otters (*Lutra canadensis*), beavers (*Castor canadensis*), and sea lions (*Zalophus californianus*), as well as species in 'damp' environments such as eastern moles (*Scalopus aquaticus*) and kinkajous (*Potos flavus*; lives in rainforest) (Lindholm and Downing, 1980; Downing and Stewart, 1987; Davis *et al.*, 1988; Stewart and Downing, 1991; Williams *et al.*, 1992; Wang *et al.*, 2018). Based on this observation, Wang *et al.*, (2018)

suggested squalene performs a function specific to mammals whose surface is often wet. However, squalene also makes up a large proportion of human sebum and is found in several species of bats (Smith and Thiboutot, 2008; Pannkuk *et al.*, 2012, 2013). Squalene is common in scent gland secretions of many land-dwelling mammals, such as pandas, peccaries, lemurs, and bats, and serves as a fixative to further extend the life of volatile compounds (Waterhouse *et al.*, 1996; Wood *et al.*, 2005; Dingzhen *et al.*, 2006; Scordato *et al.*, 2007). Convincing evidence regarding ecological patterns in sebum composition awaits the characterization of a greater variety of mammal species.

Sebum composition also varies within individuals, such as among body parts. Sebum on the surface of bat wings has more free fatty acids and sterol/wax esters than hair or wing epidermal tissue (Pannkuk *et al.*, 2012). Lipid quantity varies among body parts in California sea otters (*Enhydra lutris*) as the skin had greater abundance of lipids than the fur, and the lower back had more lipids compared to other body parts such as the head (Williams *et al.*, 1992). Lipid composition on hair varies among individuals and body parts in white-tail deer (*Odocoileus virginianus*) in terms of the quantity and occurrence of specific compounds such as decane, undecane, carvone, alkanes, arenes, ketones, aldehydes, phenols, and terpenes (Gassett *et al.*, 1997). This variation may reflect different requirements among body parts in terms of sebum functionality. It may also contribute to differences among body parts in disease occurrence such as *Malassezia*- and ectoparasite-associated skin diseases as discussed in the previous section.

A variety of factors may affect sebum quantity and composition among and within individuals of a species including hormones, season, skin pH, diet, age, and sex. Factors such as diet may also partially explain variation in sebum composition among species, but it is unknown

why sebum composition is species-specific in all taxa characterized to date, and what mechanisms drive this variation.

Hormones

In humans and laboratory mammals androgenic hormones cause an increase in sebaceous gland size by stimulating both the rate of cell division and the rate of lipid accumulation (Thody and Shuster, 1989; Stewart and Downing, 1991; Zouboulis, 2004; Makrantonaki *et al.*, 2011). The increase in androgen levels at puberty in humans causes a large increase in the rate of sebum secretion and also changes lipid composition (Stewart and Downing, 1991; Zouboulis, 2004; Makrantonaki *et al.*, 2011). In contrast, estrogens tend to inhibit sebaceous gland activity and decrease gland size (Thody and Shuster, 1989; Zouboulis *et al.*, 2016). There are several reviews that summarize the effect of various hormones on sebaceous glands in humans and laboratory mice and rats (Thody and Shuster, 1989; Smith and Thiboutot, 2008; Zouboulis *et al.*, 2016). Given these patterns, hormones likely also have a major influence on sebum production and composition in wild mammals. For instance, scent glands are enlarged and more active in males of multiple wild mammalian species during breeding season, when testosterone levels are high and oily skin secretions are apparent in areas of the body used for scent marking, and shrink during the non-breeding season (Quay, 1953; Quay and Muller-Schwarze, 1970; Clarke and Frearson, 1972; Kennaugh *et al.*, 1977; Albone, 1984; Pinter, 1985; Pandey and Dominic, 1987; Rasmussen, 1988; Stoddart and Bradley, 1991; Hardy *et al.*, 1991; Khazanehdari *et al.*, 1996; Buesching *et al.*, 2002; Wood *et al.*, 2005; Nassar *et al.*, 2008; Bakshi, 2010; Blank *et al.*, 2014; Martín *et al.*, 2014; Tomiyasu *et al.*, 2018). This pattern also occurs in the scent glands of some domesticated and laboratory animals (Jenkinson *et al.*, 1967; Thiessen, 1968; Ebling, 1977).

Injecting testosterone into females and castrates promotes the development of glands and secretions similar to mature males (Mitchell, 1965; Clarke and Frearson, 1972; Stoddart, 1972; Jannett, 1975; Balakrishnan and Alexander, 1980; Pinter, 1985; Stoddart and Bradley, 1991; Iburg *et al.*, 2013). Injecting progesterone into castrates can also increase the size and secretion rate of scent glands and increase the frequency of scent marking behavior (Balakrishnan *et al.*, 1984). However, these patterns are not seen in all wild mammalian species, such as kangaroo rats (*Dipodomys* spp.) (Quay, 1953; Randall, 1986). The effect of hormones on sebum quantity and composition between sexes and among age classes partially explains patterns of skin disease occurrence as discussed in the previous section.

Season

Sebum composition and quantity varies seasonally in multiple species. For example, the free fatty acid portion of lipids that coat porcupine (*Erethizon dorsatum*) quills is higher in summer compared to winter (Roze *et al.*, 1990). The amounts of myristic, stearic, and linoleic acids in the wing *of Myotis lucifugus* decreases over the hibernation season while pentadecanoic, palmitoleic, and oleic acids levels increase (Frank *et al.*, 2016; Ingala *et al.*, 2017). Sebaceous glands in moose (*Alces alces*) skin which are not part of specialized scent glands are reduced in winter and well developed in summer (Sokolov and Chernova, 1987). Sebum output in domesticated cattle is lower in winter compared to summer (Smith and Jenkinson, 1975), which may be caused by temperature differences. Sebum composition varied among domesticated cattle experimentally exposed to different temperatures (24ºC, 32ºC, 38ºC) over two weeks (O'Kelly and Reich, 1982). The amount of esterified fatty acids excreted in triglycerides decreased while the amount excreted in wax esters increased with rising body temperature in the Brahman cattle

breed, but not in the British breed (O'Kelly and Reich, 1982). Knowledge gaps remain regarding environmental effects on sebum composition and quantity and how these changes affect sebum function and disease susceptibility.

Skin pH

Cutaneous pH can affect sebum composition in humans and laboratory mammals, and this may also apply to wild mammals. Some free fatty acids (a component of sebum) are generated within skin from phospholipids by secretory phospholipase A_2, and this enzyme is inactivated at alkaline pH (>7 pH), partially due to the activation of serine proteases (Behne *et al.*, 2002; Fluhr *et al.*, 2004). Acidic pH is also important for direct influence of lipid–lipid interactions in the lamellar bilayers of the permeability barrier (Bouwstra *et al.*, 1999). Stratum corneum neutralization reduces competence of permeability barrier lipids (Mauro *et al.*, 1998; Hachem *et al.*, 2003). Sebum quantity and skin pH are inversely correlated in humans (Wan *et al.*, 2014).

Diet, Age, Sex

Sebum composition is affected by diet (Melnik, 2015; Lovászi *et al.*, 2018). Sebocytes synthesize all lipid classes present in sebum, but can also take up preformed lipids or remodel lipids from the bloodstream (Zouboulis *et al.*, 2016). Severe caloric restriction or fasting in humans decreases sebum quantity and changes skin surface lipid composition as triglyceride and wax ester secretion is reduced (Downing *et al.*, 1972). Young pigs fed a diet deficient in essential fatty acids develop altered skin lipid composition accompanied by scaly skin and greatly increased trans-epidermal water loss compared to pigs fed a regular diet (Melton *et al.*, 1987).

754 Dogs fed diets deficient in essential fatty acids develop seborrhoea, while supplementing their
755 diet with sunflower oil or olive oil changed skin lipid composition and ameliorated symptoms
756 (Campbell and Dorn, 1992; Campbell *et al.*, 1992). Variation in sebum composition among bat
757 species may be partially due to diet (Pannkuk *et al.*, 2012; Frank *et al.*, 2016; Ingala *et al.*, 2017).
758 The composition and quantity of human sebum varies with age and sex, but there are also
759 marked differences among individuals (Thody and Shuster, 1989; Zouboulis *et al.*, 2016). In
760 laboratory rats, sex and age-related differences in lipid composition are larger than differences in
761 fur collected from various body regions within an individual, and much larger than inter-animal
762 differences in age and sex-matched specimens (Khandelwal *et al.*, 2014).
763
764 **CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH**
765 Research on sebum function has focused on the maintenance of healthy skin and defense
766 against pathogens, while research on scent gland function has focused on chemical
767 communication. There may be more overlap in function between these two gland types than is
768 currently recognized. Non-specialized sebaceous glands may play a role in chemical
769 communication via delivery of pheromones to the skin surface, although it is unclear if this is
770 important compared to specialized scent glands (Smith and Thiboutot, 2008). If it is important,
771 this function may help explain why sebum composition is species-specific. Further studies on the
772 potential function of scent glands in skin disease occurrence and progression are also needed.
773 Mammals that lack sebaceous glands generally have no to sparse hair/fur, and laboratory
774 mice that are genetically modified to lack sebaceous glands or have inactive glands lose their fur.
775 This illustrates the importance of sebum in maintaining healthy hair/fur. However, sebaceous
776 glands are also absent in Cynocephalidae (colugos) that have fur, which suggests sebaceous

glands are not always necessary to maintain healthy fur. Further studies on the skin properties of colugos and other mammals that lack sebaceous glands may provide insights into mechanisms that replace sebum functions when sebaceous glands are absent.

It is unknown how much research on sebum function in humans and laboratory/domestic mammals applies to wild mammals given species-specific differences in sebum composition. This has already proven problematic in acne research, as traditional laboratory mammals do not normally develop acne and have different sebum compositions from humans (Schneider and Zouboulis, 2018). This problem has been partially addressed through the use of genetically modified laboratory mammals and human sebocytes *in vitro* (Schneider and Zouboulis, 2018). These techniques may also facilitate laboratory studies on sebum function in wild mammals.

A promising area of research is the effect of microbes on skin lipid composition, and vice versa, and how those effects contribute to skin defense against pathogen establishment and disease progression. Elucidating which microbes on the skin are important in generating free fatty acids or other lipids that prevent the establishment or growth of pathogens may facilitate biocontrol treatments for skin diseases. Determining which components of sebum different microbial species use for nutrition may provide insight into variation in skin microbiomes among and within individuals and species, given the wide variation in sebum composition.

Skin is an effective physical and chemical barrier to pathogens and often skin disease only results when these properties are compromised by wounds, environmental factors (such as persistent wetting), or other diseases. Sebum makes up part of this physical and chemical barrier. Some ectoparasites, fungi, and bacteria on the skin only become pathogenic when the skin environment changes, such as disruptions of the protective lipid layer (over- or under-production of sebum), immune system, skin pH, or cutaneous microbiome. Infections can change sebum

composition, which can compromise functions performed by sebum such as its antimicrobial properties and maintaining skin barrier function. Differences in sebum composition among species may help explain species-specific differences in disease susceptibility, as certain lipid components may have more effective antimicrobial functions (chemical barrier) against specific pathogens compared to other lipid components. Sebum is a physical barrier to external water (e.g., rain) and also pathogens if microbes lack enzymes capable of penetrating the sebaceous film.

Baseline data on the normal sebum composition of wild mammals are required, especially for species-rich groups like Rodentia and Chiroptera. In addition to the intrinsic interest of revealing the variety of sebum composition in uninvestigated species, such data may provide further insight on the biological roles of sebum and why sebum composition is species-specific in all taxa characterized to date. More data on sebum across taxa could clarify the roles of ecological factors and phylogeny in shaping sebum quantity and composition.

Sebum quantity and composition varies with season, sex, age, and body part in some wild mammals. Based on research in domestic mammals, diet, skin pH, and hormones are likely also important in wild mammals, but have yet to be studied. Elucidating how these factors affect sebum quantity and composition in multiple taxa may provide insight into skin disease susceptibility that can also vary with these factors.

Questions remain concerning environmental effects on sebum quantity and composition in wild mammals, including factors such as temperature, moisture, pollutants, and chemicals. Climate change, which encompasses increasing exposure to U.V. radiation and temperature changes, and exposure to pollutants may have unanticipated effects on skin physiology and

barrier function. If these factors change sebum composition or output, it may affect disease susceptibility, possibly in a species-specific manner.

Sebaceous gland perturbation, especially alterations in lipid synthesis and composition, may be an under recognized contributor in the pathogenesis of skin diseases of wild mammals. Additional studies are needed to further assess and clarify the contribution of sebaceous glands to skin maintenance and defense, particularly as new wildlife skin diseases are discovered. Such studies may uncover new therapeutic strategies for the treatment of skin diseases.

Table 5.1: Summary of available literature describing the sebum composition of live, wild mammals. When more than one species of mammal was studied within a citation, we separated each species record, so some papers are represented more than once. Studies with a * also contain data on domestic or laboratory mammals. A study that analyzed the lipid composition of fur from dead mammals (road kill and skins in collections) was excluded (Lindholm et al., 1981), as lipid composition likely changes after death.

Reference	Country	Months Samples Collected	Captivity Status	Species	Common Name	Sex	Age	n	Sample Type	Sebum or Epidermal lipids
(Frank *et al.*, 2016)	United States	Winter Winter, October, March	Free-ranging	*Eptesicus fuscus* *Myotis lucifugus*	Big brown bat Little brown myotis	Unknown	Adult	6 25	Skin biopsy	Both
(Pannkuk *et al.*, 2012)	United States	Unknown	Unknown Free-ranging	*Eptesicus fuscus* *Lasiurus borealis* *Nycticeius humeralis*	Big brown bat Eastern red bat Evening bat	Unknown	Adult	unknown	Fur, wing-skin biopsy, scrubbing skin with cotton balls	Both
(Pannkuk *et al.*, 2015)	Canada	Winter	Captive	*Myotis lucifugus*	Little brown myotis	Unknown	Adult	6	Skin biopsy	Both
(Pannkuk *et al.*, 2014a)	United States	July	Free-ranging	*Lasiurus borealis*	Eastern red bat	both sexes	Adult	5 pooled samples from 50-75 individuals	hair clipped and lipids extracted	Sebum
(Pannkuk *et al.*, 2013)	United States	Unknown	Free-ranging	*Eptesicus fuscus* *Lasiurus borealis* *Nycticeius humeralis*	Big brown bat Eastern red bat Evening bat	Unknown	Unknown	Unknown	hair, wing surface, wing tissue	Both
(Pannkuk *et al.*, 2014b)	United States	Unknown	Free-ranging	*Lasiurus borealis* *Lasiurus cinereus* *Eptesicus fuscus* *Nycticeius humeralis* *Myotis lucifugus*	Eastern red bat Hoary bat Big brown bat Evening bat Little brown myotis	Unknown	Adults & juveniles Unknown	10 adults, 10 juveniles 6 12 17 5	Sebutape adhesive patches pressed to skin	Sebum

				Myotis austroriparius	Southeastern myotis			11		
				Myotis septentrionalis	Northern long-ear bat			11		
				Myotis leibii	Small-footed bat			16		
				Myotis grisescens	Gray bat			10		
				Lasionycteris noctivagans	Silver-haired bat			6		
				Perimyotis subflavus	Tricolored bat			9		
				Corynorhinus rafinesquii	Rafinesque's big-eared bat			11		
				Corynorhinus townsendii ingens	Ozark big-eared bat			5		
(Řezanka *et al.*, 2015)	Czech Republic	spring	Free-ranging	*Myotis myotis*	Greater mouse-eared bat	Male, Female	Adult	6 Male, 6 Female	lipids isolated with chloroform from clipped fur	Sebum
(Downing and Stewart, 1987)	United States	April	Free-ranging	*Scalopus aquaticus*	Eastern mole	Unknown	Unknown	1	Body dipped in acetone	Sebum
(Colton and Downing, 1983) *	Unknown	Unknown	Unknown	*Equus przewalskii*	Przewalski's horse	Unknown	Unknown	Unknown	Acetone poured on skin & then scraped off	Sebum
				Equus grevyi	Grevy's zebra					
				Equus hemionus	Onager					
(Roze *et al.*, 1990)	United States	August, January, February	Free-ranging	*Erethizon dorsatum*	Porcupine	Unknown	Unknown	7	Quills	Sebum
(Wix *et al.*, 1987) *	United States	Unknown	Unknown	*Erethizon dorsatum*	Porcupine	Unknown	Unknown	1	hair, quills	Sebum

				Macaca fascicularis	Crab-eating macaque			1	hair	
(Nishimaki-Mogami *et al.*, 1988)	Japan	Unknown	Unknown	*Macaca fascicularis*	Crab-eating macaque	Male	Unknown	3	Shaved skin wiped with acetone	Sebum
(Birkby *et al.*, 1982) *	Unknown	Unknown	Unknown	*Procyon lotor*	Racoon	Unknown	Unknown	1	Hair	Sebum
				Macaca fascicularis	Crab-eating macaque			1		
(Nicolaides *et al.*, 1968) *	United States	Unknown	Captive	*Pan troglodytes* species unknown	Chimpanzee	Unknown	Unknown	1	hair clipped, skin washed with hexane	Sebum
					Baboon			1		
(Gassett *et al.*, 1997)	United States	December	Captive	*Odocoileus virginianus*	White-tailed deer	Male	1.5-11.5 years	10	hair	Sebum
(Colton *et al.*, 1986)	United States	Unknown	Unknown	*Neogale vison*	Mink	Female	Unknown	2	Acetone poured over mid-section	Sebum
(Waldorf and Vedros, 1978)	United States	August	Free-ranging	*Callorhinus ursinus*	Northern fur seal	Male	Adult	8	acetone poured on skin	Sebum
(Williams *et al.*, 1992)	United States	Unknown	Unknown	*Enhydra lutris*	California sea otter	Unknown	Adult	1	fur, skin biopsy	Both
(Davis *et al.*, 1988)	United States	Summer	Captive	*Enhydra lutris*	California sea otter	Male	Unknown	8	Fur	Sebum
				Lutra canadensis	Otter			1		
(Lindholm and Downing, 1980)	United States	Unknown	Captive	*Castor canadensis*	Beaver	Unknown	Unknown	1	Fur	Sebum
				Potos flavus	Kinkajou			2		

Table 5.1: continued

Reference	Method	Body Region	Conclusion

(Frank *et al.*, 2016)	gas-liquid chromatograph	Wings	fatty acid composition differed between *Eptesicus fuscus* and *Myotis lucifugus* as the latter had more stearic acid and less palmitoleic, myristic, and oleic acid than the former. wing lipids of *M. lucifugus* prior to hibernation had higher myristic, stearic, and linoleic acid levels than during late hibernation
(Pannkuk *et al.*, 2012)	thin-layer chromatography	Wings and back	Triacylglycerol proportions were higher in hair versus wing tissue. Triacylglycerol profiles were different among the 3 bat species. Bats had greater amounts of cholesterol and less squalene than humans
(Pannkuk *et al.*, 2015)	electrospray ionization tandem mass spectrometry	Wings	wing tissue from bats with white-nose syndrome had different glycerophospholipid class composition from healthy tissue
(Pannkuk *et al.*, 2014a)	liquid chromatography electrospray ionization tandem mass spectrometry	Unknown	targeted glycerophospholipids, found 152 types
(Pannkuk *et al.*, 2013)	thin-layer chromatography	Wings and back	bat sebum has a higher proportion of sterols and free fatty acids compared to human sebum, and a lower proportion of squalene and monoacylglycerides red bats have lower ratios of free fatty acids compared to big brown bats and evening bats
(Pannkuk *et al.*, 2014b)	Gas Chromatography/Mass Spectrometry	Dorsal plagiopatagium	Broad lipid classes did not differ between hair and wing, but ratios of specific free fatty acids, monoacylglycerides, squalene, and sterol differed The ratios of fatty acid methyl acid types differed between *Lasiurus borealis* adults and juveniles
(Řezanka *et al.*, 2015)	high-resolution mass spectrometry	Unknown	characterized fatty acid methyl esters which were similar among species the composition of wax esters but not steryl esters differed between the sexes
(Downing and Stewart, 1987)	thin-layer chromatography	Full body up to neck	skin lipids consisted of squalene, wax esters, and sterol esters with small amounts of triglycerides, free fatty acids, free sterols, free fatty alcohols
(Colton and Downing, 1983) *	thin-layer chromatography	Unknown	skin lipids consisted of cholesterol, cholesteryl esters, lactones, wax diesters
(Roze *et al.*, 1990)	gas chromatograph-quadrupole mass spectrometry, thin-layer chromatography	Back and tail	Lipids on quills had more free fatty acids in August compared to January/February. The free fatty acids inhibited growth of 6 of 10 bacteria strains tested.
(Wix *et al.*, 1987)*	thin-layer chromatography	Unknown Unknown	lipids consisted of ceramides, cholesteryl sufate, glycosylceramides
(Nishimaki-Mogami *et al.*, 1988)	thin-layer chromatography	Back	lipids consisted of sterol esters, cholesterol, wax diesters
(Birkby *et al.*, 1982) *	thin-layer chromatography	Unknown	contain ceramides, polar glycolipids, cholesterol, free fatty acids contain ceramides, polar glycolipids, cholesterol, free fatty acids

			contain ceramides, polar glycolipids, phospholipids
(Nicolaides *et al.*, 1968) *	thin-layer chromatography	back	lipid composition differs among species in the presence of squalene, sterol esters, wax esters, triglycerides, free fatty acids, and diesters
(Gassett *et al.*, 1997)	gas chromatography–mass spectrometry	forehead, back	only volatiles were analyzed; composition varied among individuals (e.g. different concentrations of decane, carvone, and undecane) and between the forehead and back (e.g. different concentrations of terpene, octanal, and naphthalene)
(Colton *et al.*, 1986)	thin-layer chromatography	mid-section	wax monoesters are the most common non-polar lipid on mink skin
(Waldorf and Vedros, 1978)	gas-liquid chromatography	Unknown	only fatty acid components identified; some were fungiostatic to dermatophytes *in vitro*
(Williams *et al.*, 1992)	gas/liquid & thin-layer chromatography, some by gas chromatography-mass spectrometry	Dorsal body, head, tail	lipid quantity varies among body parts (e.g. greater abundance on lower back versus head). Squalene is the principal lipid component of both fur and skin with minor amounts of other compounds such as triglycerides and cholesterol.
(Davis *et al.*, 1988)	gas chromatography	Thorax, abdomen	only squalene examined; mean 3.7 1.1 mg/g of fur. Exposure to crude oil did not change squalene concentration, but cleaning fur with Dawn soap removed it completely. Concentrations returned to base-line levels 7 days after cleaning in unoiled but not oiled fur
(Lindholm and Downing, 1980)	thin-layer chromatography	Unknown	lipid quantity: 14mg/g of fur; found squalene, glyceryl ether diesters, unidentified polar lipid, free sterol, wax esters and diesters
			lipid quantity: 3mg/g of fur; found squalene, unidentified polar lipid, free sterol, wax esters and diesters
			lipid quantity: 30mg/g of fur; found squalene, unidentified polar lipid, free sterol, wax esters and diesters

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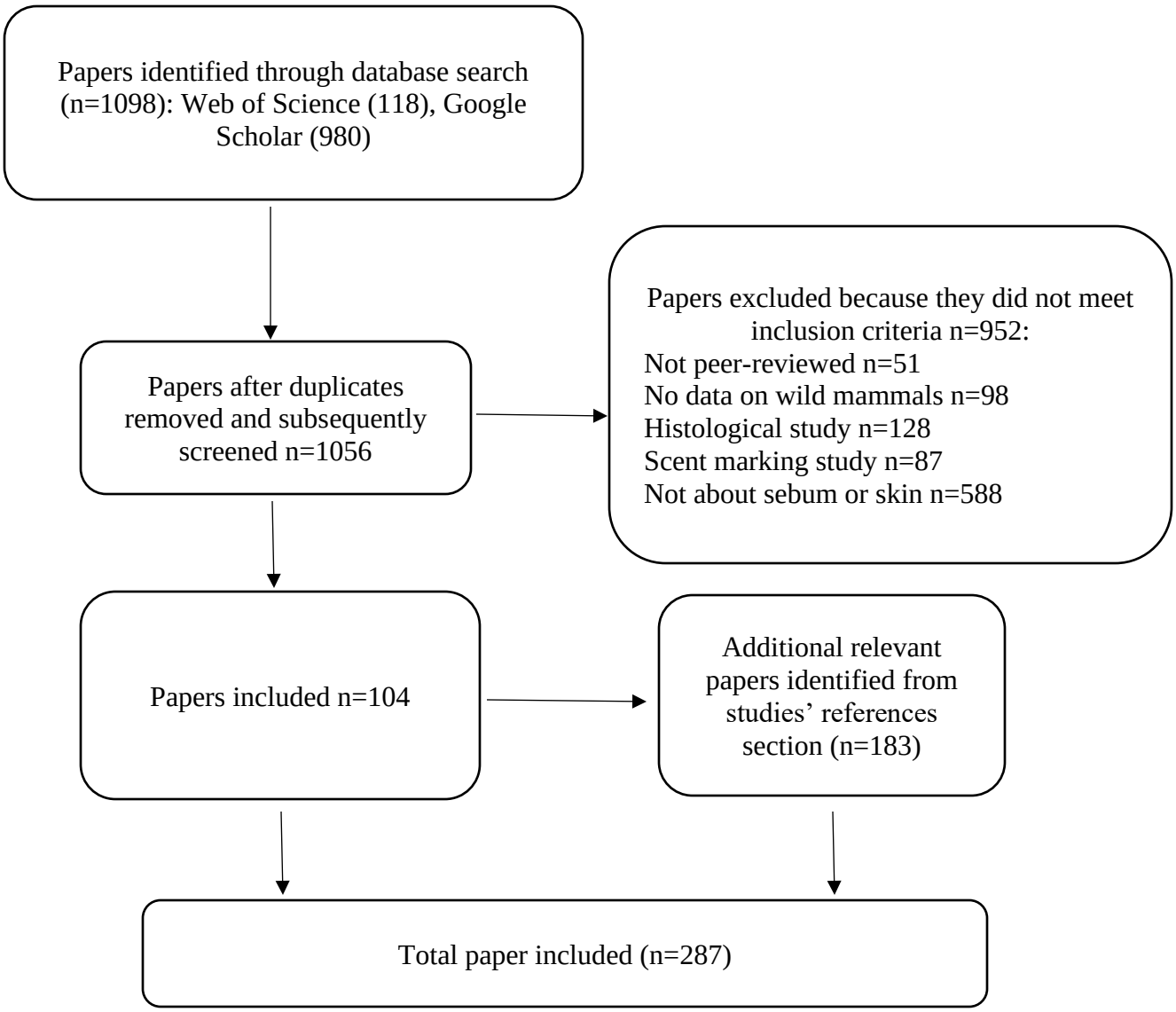


Figure 5.1: Protocol for screening articles after database search. Each step shows the number of papers included or excluded for review.

ACKNOWLEDGEMENTS

Thank you to Dr. Donald McAlpine for helpful discussions and comments on the draft.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CHAPTER 6

General Discussion

INTRODUCTION

White-nose syndrome (WNS) is an ongoing threat to various bat species in North America. Although much has been learned since the emergence of the disease in 2006, many knowledge gaps remain. This is partly due to the novelty of the disease, as deadly fungal infections of the skin are unusual among wild mammals. Each chapter of this thesis aimed to increase knowledge of the biotic and abiotic environment on the surface of mammal skin, especially bats. Understanding the mechanisms that enable some bat species to tolerate or resist infection altogether after exposure to *Pseudogymnoascus destructans* (*Pd*) is essential to the development of interventions to prevent or treat WNS. I set out to: 1) determine if skin mycobiome composition and abundance varied with species-level WNS-susceptibility, and 2) examine aspects of skin physiology, namely pH and sebum, that may influence microbial composition on the skin and disease outcomes. To address these objectives, I characterized the mycobiomes of 848 individual bats belonging to 20 species (chapters 2 and 3) and the skin pH of 710 wild bats of five species and 126 captive bats of one species (chapter 4) during the completion of this thesis. WNS-susceptible bat species had different mycobiome composition (chapter 2) and skin pH (chapter 4) compared to WNS-resistant and tolerant species. I predicted that most bat species in western North America will be WNS-susceptible based on comparing their skin mycobiomes to bat species of known susceptibility in the east (chapter 3). Some fungi isolated from bats inhibited *Pd in vitro* but only under specific salinity and pH conditions (chapters 2 and 3). This pattern illustrates the importance of abiotic conditions on the skin surface in influencing microbial interactions. Variation in sebum (lipids on the skin) quantity and

composition among species, individuals, and body parts may partially explain variation in skin microbiomes and skin disease susceptibility (chapter 5). The influence of nutrient composition, pH, and other abiotic factors on microbial interactions is well-recognized and studied in soil systems but not in skin microbiome studies, particularly non-human studies. This is demonstrated by the dearth of skin physiology studies in wild mammals (chapters 4 and 5). My thesis illustrates that understanding the physical and chemical landscape of skin is essential for understanding mechanisms structuring skin microbial assemblages and skin disease susceptibility in wildlife. In the case of bats and WNS, my thesis revealed that the skin mycobiome of bats is dominated by yeasts (just like humans, but with different yeast species) and varies with species-level WNS-susceptibility, interactions between *Pd* and other microbes are influenced by the environment, and bat skin pH varies among species and seasons which may influence WNS progression.

FUTURE DIRECTIONS

As with all research, my thesis leads to more questions that might be pursued to continue to enhance our understanding of bat life history, bats and WNS, and skin and disease in wildlife.

Skin microbiomes and disease outcomes

Based on the results of chapters two and three, further investigation of the role of bat microbiomes and specific microbial taxa in regulating disease outcomes in bats is warranted. A major barrier in the development of microbial biocontrols for WNS is determining whether *in vitro* results are applicable *in vivo*. This is because the function of cells in their native habitat

often cannot be reliably predicted from genomic data or from physiology studies of isolates *in vitro* (Hatzenpichler *et al.*, 2020). For example, I found the skin pH of bats during the winter was alkaline, which means the fungal species that inhibited *Pd in vitro* in chapters two and three likely do not do so *in vivo* because inhibition only occurred under acidic conditions. Many factors affect the outcomes of microbial interactions, including abiotic and biotic variables such as nutrient availability and composition, pH, and composition of other microbes present in the environment (Boxberger *et al.*, 2021). A better understanding of the microenvironment present on bat skin will facilitate the development of laboratory models that attempt to mimic this environment. Logistical and ethical issues limit the use of captive bats in experimental work, so laboratory models, such as skin explants or 3D models (Boxberger *et al.*, 2021), are required. However, after initial research with laboratory models, using captive bat colonies to test whether potential biocontrol microbes can be successfully established as part of the commensal microbiome on bat skin would be beneficial. If successful, challenge experiments with *Pd* could be performed to test whether bats with a specific microbiome composition are better able to fend off the pathogen as assessed by fungal load and severity of WNS clinical signs.

In this thesis, I employed a culture-based approach to examine the mycobiome of bats, which has several limitations. Specifically, taxa that are rare, cryptic, fastidious, or unculturable may be overlooked. I also swabbed one section of one wing for fungi, as opposed to the whole bat, which likely means some fungi on the bat were not detected. In humans, individuals harbor similar microbial assemblages on the left and right sides of their bodies (Ross et al. 2017) but it is unknown if this pattern also occurs in bats. Multiple studies have examined the effect of different methods on detected composition and abundance of skin microbiomes. Neither storage temperature or duration (up to two weeks) affected bacterial abundance and composition from

swabs of human skin and soil (Lauber et al. 2010). Body part explained more of the variation in human skin bacteria biodiversity compared to collection method and especially storage conditions of the samples (Manus et al. 2022). How well microbes adhere to a surface affects the likelihood of being picked up by a swab (Nobbs et al. 2011), and the type of swab used can also influence results (Horn et al. 2008). Using consistent methods is important to ensure samples are comparable. More research on the effect of different methods on the results of skin microbiome research in wildlife are needed.

Fully characterizing skin microbial assemblages by assessing the structure or composition of its members is challenging due to the dynamic nature and functional redundancy within microbial communities. For this reason, microbial ecology studies have begun to focus on functional characteristics rather than exclusively on taxonomy and phylogeny (Hatzenpichler *et al.*, 2020). Further insight into microbial-mediated suppression of *Pd* by native microbiomes may be gained by screening for anti-fungal metabolites using liquid chromatography–mass spectrometry or a genomics approach to characterize functional diversity. This approach may reveal mechanisms of pathogen suppression and help determine whether pathogen-suppressive functions are an emergent property of microbial assemblages or can be attributed to an individual species.

Effect of skin physiology on skin microbiomes and disease outcomes

If the skin microbiomes of bats moderate infection and disease, then identifying factors that determine the composition and structure of the microbiome is critical for understanding the epidemiology of this resistance and how we might enhance survival rates within a population.

Mechanisms driving inter-specific differences in skin microbiomes likely include aspects of skin physiology such as pH and lipid composition in addition to environmental effects.

Host and local environmental factors appear to interact closely to shape bat-skin microbiomes, with conditions on the skin favoring particular taxa from an environmental reservoir, as has been suggested for amphibian microbiomes (Walke *et al.*, 2014). I found in chapters two and three that site is an important factor explaining variation in bat-skin microbiomes, but microbiomes also vary among individuals within a site. Chemical gradients drive the ecology and physiology of microbial communities in soil, sediment, and other microbial ecosystems (Quinn *et al.*, 2018), and likely also do so on skin surfaces. Identifying parameters that influence the diversity, composition, and functionality of bat-skin microbiomes may provide insight into varying levels of WNS-susceptibility, as *Pd* is part of bats' microbiome, as well as susceptibility to other skin diseases, such as dermatitis. These parameters, such as pH and sebum, may have synergistic effects. Skin physiology may affect disease outcomes indirectly by influencing commensal microbiome composition, microbial interactions on the skin, and skin functions such as cutaneous water loss or immune responses, and directly by affecting the ability of pathogens to attach, grow, and produce virulence factors on the skin.

Skin microbiomes include fungi, bacteria, viruses, archaea, and invertebrates (Chen et al. 2018, Boxberger et al. 2021). These different groups can interact with each other either negatively, such as through competition for space and resources or by releasing antimicrobial compounds, or positively, for example by modifying the skin environment such that it is more favorable for other species (Oever and Netea 2014, Byrd et al. 2018, Chen et al. 2018). Modifying the skin environment can include altering the pH, changing the composition of available nutrients, such as by hydrolyzing components of sebum, or excluding certain microbes

(my enemy's enemy is my friend) (Drake et al. 2008, Byrd et al. 2018, Chen et al. 2018). These potential interactions have not yet been studied in bats except for *in vitro* studies of bacteria from bat skin and *Pd* (Hoyt et al. 2015, Hamm et al. 2017, Li et al. 2021). Like skin physiology, the skin microbiome (in its entirety) may have affected the results of my mycobiome study in chapters two and three.

Rates of cutaneous water loss, which are positively correlated with skin lipid quantity and skin pH, is a barometer to assess skin barrier functionality in humans and domestic mammals (Ohman and Vahlquist, 1994; Muñoz-Garcia *et al.*, 2012). Dehydration is thought to be a major mechanism leading to WNS-related death (Cryan *et al.*, 2013). *Eptesicus fuscus* are known to tolerate lower humidity than *M. lucifugus* and roost in different microclimates during hibernation (Klüg-Baerwald and Brigham, 2017), but the mechanism of this tolerance is unknown. Bat species that can tolerate low humidity levels during hibernation, such as *E. fuscus*, may have more skin lipids, or a different composition of skin lipids that limits cutaneous water loss, than species that are intolerant of low humidity, such as *M. lucifugus*. Differences in the ability of bats to prevent dehydration may partly explain why some bats are more tolerant of WNS.

Elucidating the mechanisms by which aspects of skin physiology influence the composition and functionality of microbiomes may indicate potential methods for manipulating the microbiome. For example, skin pH can be temporarily modified with topical products and by different bat-roost substrates. Altering skin pH may change the rate of cutaneous water loss or the functionality of enzymes produced by microbes on the skin (including *Pd*), which may also change WNS-associated mortality and morbidity. Removing sebum from the skin may render bats more susceptible to WNS, while augmenting components of sebum on the skin, such as free

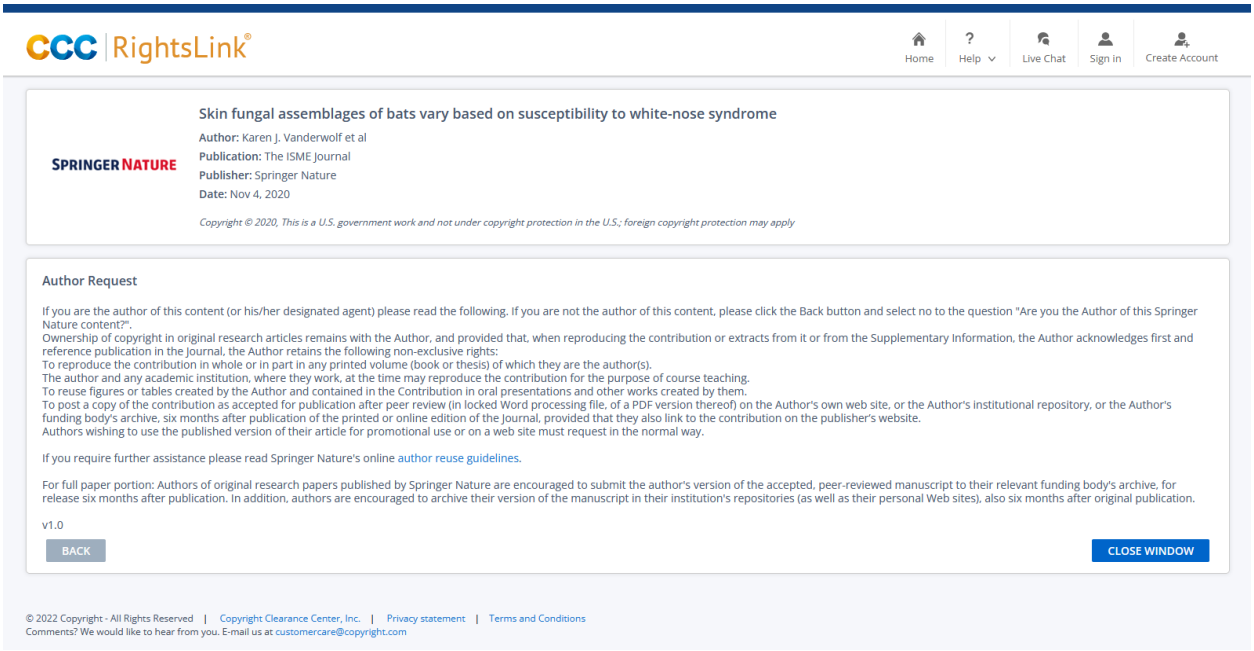
fatty acids, may make bats less susceptible. The effects of temporarily changing aspects of skin physiology could be tested with *Pd*-challenge experiments in a captive bat colony.

CONCLUSIONS

Outcomes of the work completed for this thesis further our understanding of inter- and intra-specific differences among bat species and individuals in skin mycobiomes and physiology that may contribute to variation in WNS-susceptibility. I also reviewed current knowledge on aspects of skin physiology of wild mammals, specifically sebum and pH, which clarifies patterns among different taxa and highlights knowledge gaps. The WNS research community has benefited from past research on chytridiomycosis, a deadly fungal skin infection of amphibians, and it is therefore expected that knowledge gained from bats will inform future research in other taxa as new skin diseases emerge.

APPENDIX – PERMISSIONS

Chapter 2



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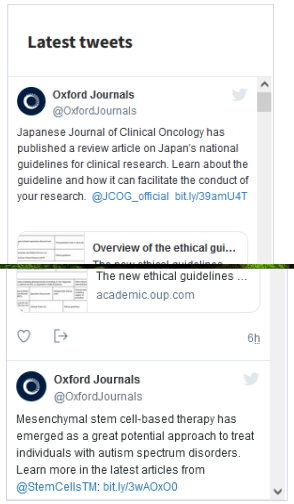

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APPENDIX – Supplemental Data

Chapter 2 Supplemental Data

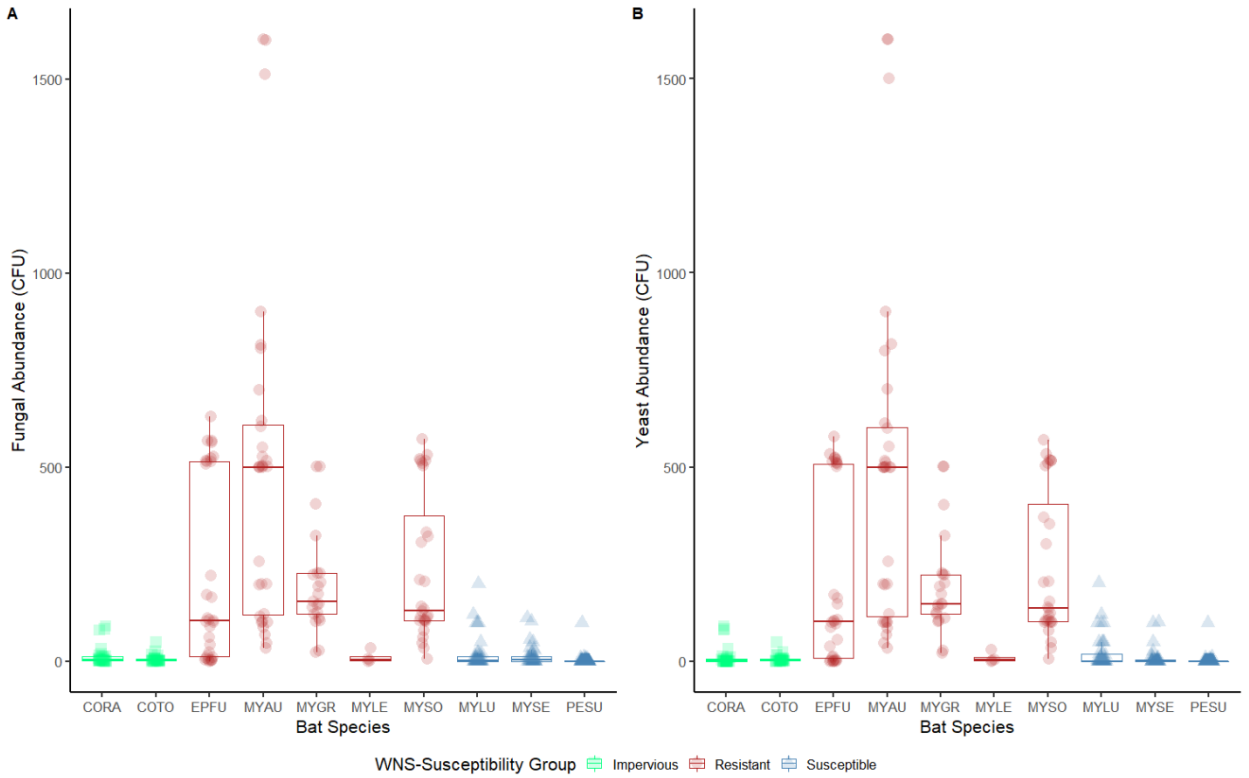

Figure 2.S1: Mean counts, expressed as colony forming units (CFU) for all fungi (A) and yeast only (B) based on white-nose syndrome (WNS)-impervious, WNS-susceptible, and WNS-resistant species of bats. CORA= *Corynorhinus rafinesquii*, COTO= *C. townsendii virginianus*, EPFU= *Eptesicus fuscus*, MYAU= Myotis austroriparius, MYGR= *M. grisescens*, MYLE= *M. leibii*, MYSO= *M. sodalis*, MYLU= *M. lucifugus*, MYSE= *M. septentrionalis*, and PESU= *Perimyotis subflavus*.

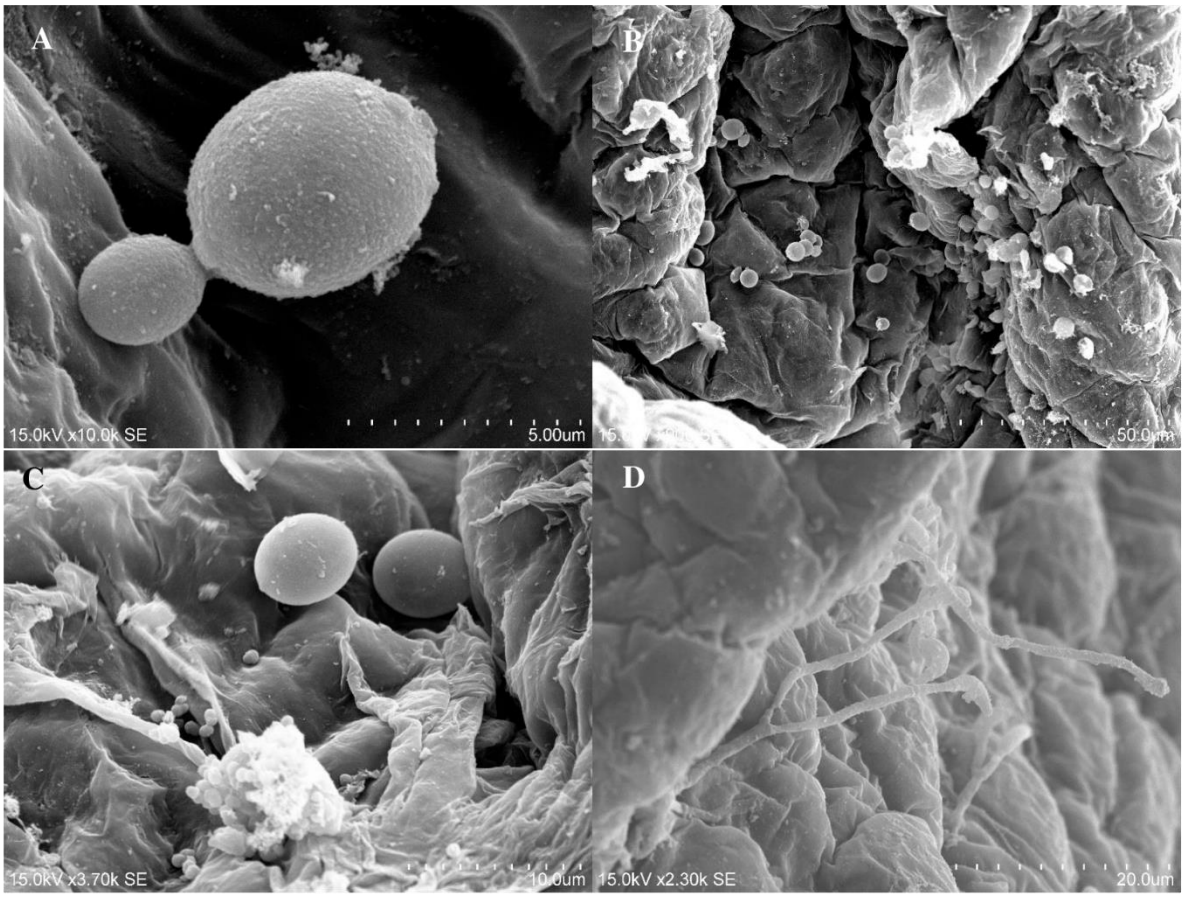

Figure 2.S2: Images A – C: Yeast cells on the wing surface of an *Eptesicus fuscus* sampled live from a hibernaculum in Wisconsin. Image C also shows smaller bacteria cells. Image D: Hyphae and conidia of *Pseudogymnoascus destructans* on the wing surface of a *Myotis lucifugus* sampled live from a hibernaculum in Wisconsin. Images were taken under high-vac scanning electron microscopy. Scale bar numbers represent the full length of the bar.

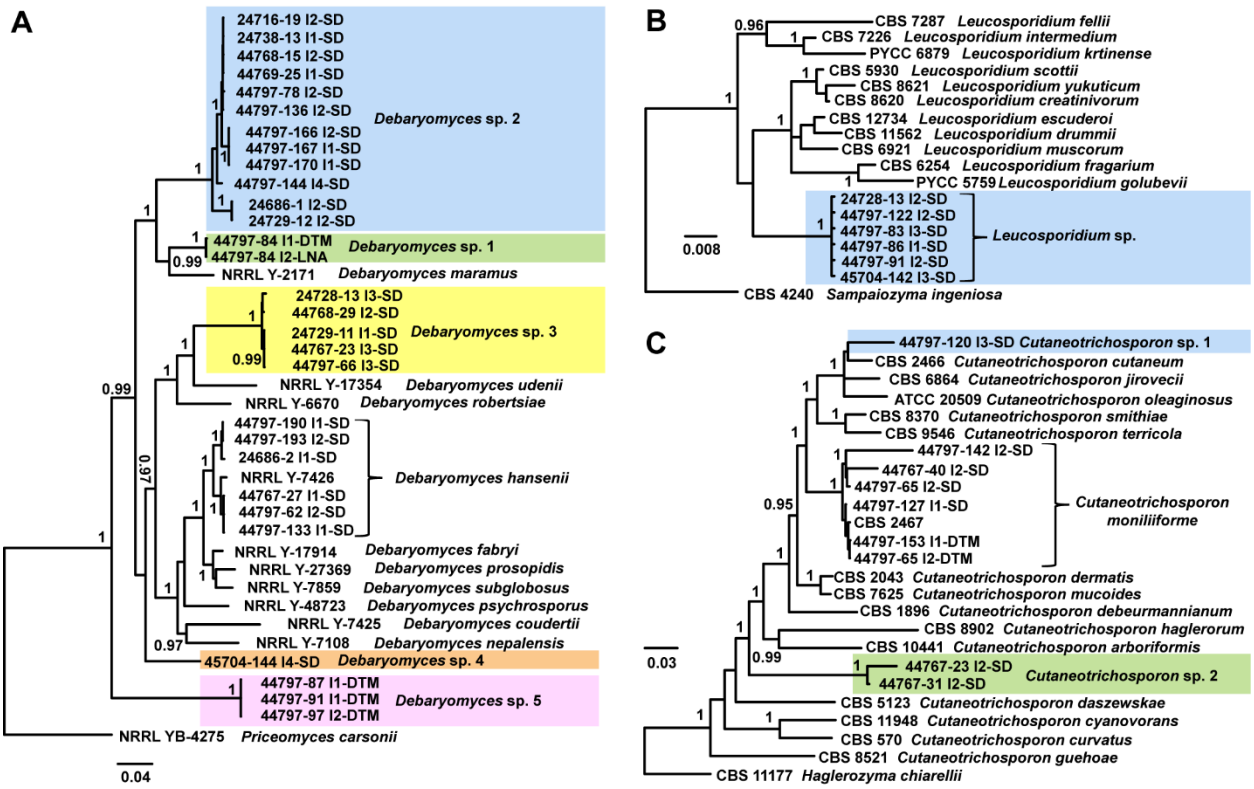

Figure 2.S3: Phylogenetic trees from Bayesian analyses of concatenated nucleotide sequences of multiple loci for fungal taxa that were differentially more abundant on bat species that are resistant or impervious to white-nose syndrome. Posterior probabilities are presented at each node if they were greater than 0.95. Type strains for each species of the genus are listed with their associated culture collection identifiers. Potentially novel taxa (as determined by genetic divergence from described species) isolated in this study appear in shaded boxes. (A) Phylogenetic tree for the genus *Debaryomyces* using five loci (internal transcribed spacer [ITS], the D1/D2 region of 26S rDNA [D1D2], second largest subunit of RNA polymerase II [RPB2], actin, and mitochondrial cytochrome c oxidase subunit II); (B) phylogenetic tree for the genus *Leucosporidium* using two loci (ITS, D1D2); and (C) phylogenetic tree for the genus *Cutaneotrichosporon* using four loci (ITS, D1D2, RPB2, translation elongation factor 1-α).

Table 2.S1: Fungal taxa cultured from the wing surface of bats in the eastern United States. Numbers indicate the number of individual bats from which each fungal operational taxonomic unit (OTU) was cultured. Fungal taxa indicated by * were identified using NCBI BLAST function; all other taxa were identified using UNITE. "NA" indicates the OTU could not be identified with available databases. CORA= *Corynorhinus rafinesquii*, COTO= *C. townsendii* virginianus, EPFU= *Eptesicus fuscus*, MYAU= *Myotis austroriparius*, MYGR= *M. grisescens*, MYLE= *M. leibii*, MYSO= *M. sodalis*, MYLU= *M. lucifugus*, MYLUr= *M. lucifugus* from WNS-resistant colonies in New York, MYSE= *M. septentrionalis*, and PESU= *Perimyotis subflavus*.

Phylum	Class	Order	Family	Genus

Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*
Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*
Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*
Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Acrodontium*
Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Acrodontium*
Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Acrodontium*
Ascomycota	Dothideomycetes	Dothideales	Aureobasidiaceae	*Aureobasidium*
Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	*Dothiora* 98%*
Ascomycota	Dothideomycetes	*Incertae sedis*	Eremomycetaceae	*Arthrographis*
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*NA*
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*
Ascomycota	Eurotiomycetes	Chaetothyriales	Cyphellophoraceae	*Cyphellophora*
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Phialophora*
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
Ascomycota	Eurotiomycetes	Onygenales	Arthrodermataceae	*Arthroderma*
Ascomycota	Eurotiomycetes	Onygenales	Arthrodermataceae	*Arthroderma*
Ascomycota	Eurotiomycetes	Onygenales	Arthrodermataceae	NA
Ascomycota	Eurotiomycetes	Onygenales	Arthrodermataceae	NA
Ascomycota	Eurotiomycetes	Onygenales	Gymnoascaceae	*Gymnoascus*
Ascomycota	Eurotiomycetes	Onygenales	Gymnoascaceae	*Gymnoascus* 99%*
Ascomycota	Eurotiomycetes	Onygenales	NA	NA
Ascomycota	Eurotiomycetes	Onygenales	NA	NA
Ascomycota	Eurotiomycetes	Onygenales	Onygenaceae	*Auxarthron*
Ascomycota	Eurotiomycetes	Onygenales	Onygenaceae	*Auxarthron*
Ascomycota	Eurotiomycetes	Onygenales	Onygenaceae	NA
Ascomycota	Eurotiomycetes	Onygenales	Onygenaceae	NA
Ascomycota	Eurotiomycetes	Onygenales	*Incertae sedis*	*Chrysosporium*
Ascomycota	Eurotiomycetes	Onygenales	*Incertae sedis*	*Chrysosporium*
Ascomycota	Eurotiomycetes	Onygenales	*Incertae sedis*	*Chrysosporium*
Ascomycota	Eurotiomycetes	Onygenales	*Incertae sedis*	*Chrysosporium*
Ascomycota	Eurotiomycetes	Onygenales	*Incertae sedis**	*Chrysosporium* 98%*
Ascomycota	Eurotiomycetes	Onygenales	*Incertae sedis**	*Chrysosporium* 99%*
Ascomycota	Eurotiomycetes	Onygenales*	*Incertae sedis**	*Chrysosporium* 99%*
Ascomycota	Eurotiomycetes*	Onygenales*	Onygenaceae*	NA
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	*Mollisia*
Ascomycota	Leotiomycetes	Helotiales	*Incertae sedis*	*Cadophora*

Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	NA
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*
Ascomycota	Leotiomycetes	Helotiales	NA	NA
Ascomycota	Leotiomycetes	Helotiales	NA	NA
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	*Botrytis* 97%*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Geomyces*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudeurotium*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudogymnoascus*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudogymnoascus*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudogymnoascus*
Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	NA
Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	*Retiarius*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Yamadazyma*
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Kluyveromyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Torulaspora*
Ascomycota	Saccharomycetes	Saccharomycetales	*Incertae sedis*	*Candida*
Ascomycota	Saccharomycetes	Saccharomycetales	Trichomonascaceae	*Trichomonascus*
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	NA
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Verticillium*
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Verticillium*
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Verticillium*
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Metacordyceps*
Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	*Lecanicillium*
Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	*Lecanicillium*
Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	NA
Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	NA
Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	*Simplicillium*
Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	*Simplicillium*

Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	*Simplicillium*
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma*
Ascomycota	Sordariomycetes	Hypocreales	*Incertae sedis*	*Sarocladium*
Ascomycota	Sordariomycetes	Hypocreales	NA	NA
Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Acaulium*
Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Cephalotrichum*
Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Gamsia*
Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Gamsia*
Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Scopulariopsis*
Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Wardomyces*
Ascomycota	Sordariomycetes	Ophiostomatales	Ophiostomataceae	*Sporothrix*
Ascomycota	Sordariomycetes	Ophiostomatales	Ophiostomataceae	*Sporothrix*
Ascomycota	Sordariomycetes	Sordariales	Cephalothecaceae	*Cryptendoxyla*
Ascomycota	Sordariomycetes	Sordariales	Cephalothecaceae	*Phialemonium*
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Humicola*
Ascomycota	Sordariomycetes	Xylariales	Sporocadaceae	*Neopestalotiopsis*
Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	*Entosordaria* 88%*
Basidiomycota	Agaricostilbomycetes	Agaricostilbales	Kondoaceae	*Kondoa*
Basidiomycota	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae	*Cystobasidium*
Basidiomycota	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae	*Cystobasidium*
Basidiomycota	Cystobasidiomycetes	*Incertae sedis*	Symmetrosporaceae	*Symmetrospora*
Basidiomycota	Cystobasidiomycetes	Erythrobasidiales	Erythrobasidiaceae	*Erythrobasidium*
Basidiomycota	Exobasidiomycetes	Microstromatales	*Incertae sedis*	*Pseudomicrostroma*
Basidiomycota	Malasseziomycetes	Malasseziales	Malasseziaceae	*Malassezia*
Basidiomycota	Malasseziomycetes	Malasseziales	NA	NA
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae	*Leucosporidium*
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Cystofilobasidium*
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Guehomyces*
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Udeniomyces*
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*
Basidiomycota	Tremellomycetes	Holtermanniales	*Incertae sedis*	*Holtermanniella*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Hannaella*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Hannaella*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*
Basidiomycota	Tremellomycetes	Tremellales	Cryptococcaceae	*Kwoniella*
Basidiomycota	Tremellomycetes	Tremellales	Cryptococcaceae	*Kwoniella*
Basidiomycota	Tremellomycetes	Tremellales	Rhynchogastremataceae	*Papiliotrema*
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	*Cryptococcus*

Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Apiotrichum*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Trichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Vanrija*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mucoromycota	Mucoromycetes	Mucorales	Mucoraceae	*Helicostylum*
Mucoromycota	Mucoromycetes	Mucorales	Mucoraceae	NA
Mucoromycota	Mucoromycetes	Mucorales	Mucoraceae	NA

Table 2.S1 continued

Species	Total Bats	CORA	COTO	EPFU	MYAU	MYGR	MYLE
delicatulum	29	0	0	4	9	2	0
halotolerans	1	0	0	1	0	0	0
NA	2	0	0	1	0	1	0
crateriforme	1	0	0	0	0	0	0
crateriforme	3	0	0	3	0	0	0
crateriforme	1	0	0	0	0	0	0
pullulans	8	2	0	1	1	1	0
NA	1	0	0	0	1	0	0
NA	1	0	0	1	0	0	0
NA	1	0	0	0	0	0	0
NA	1	0	0	0	0	0	0
NA	3	0	0	0	0	0	0

hyalina	1	0	0	1	0	0	0
bialowiezense	1	0	0	1	0	0	0
corylophilum	2	1	0	1	0	0	0
NA	27	0	0	10	5	1	1
NA	1	0	0	0	0	0	1
NA	15	0	0	4	4	0	0
toxicarium	1	0	0	0	1	0	0
insingulare	1	0	0	0	1	0	0
NA	2	0	0	0	0	0	0
NA	1	0	0	1	0	0	0
NA	1	0	0	1	0	0	0
NA	2	0	0	0	2	0	0
NA	1	0	1	0	0	0	0
NA	1	0	0	0	0	0	0
NA	1	0	0	0	0	0	0
thaxteri	2	0	0	1	0	1	0
umbrinum	1	0	0	1	0	0	0
NA	1	0	0	1	0	0	0
NA	2	2	0	0	0	0	0
NA	1	0	0	0	0	0	1
NA	3	0	0	3	0	0	0
NA	1	0	0	0	0	0	1
vallenarense	1	0	0	0	0	0	0
NA	1	0	1	0	0	0	0
NA	2	0	1	1	0	0	0
NA	1	0	0	0	0	0	0
NA	4	0	0	0	0	1	0
NA	1	1	0	0	0	0	0
gregata	1	0	0	0	0	0	0
NA	1	0	0	0	0	0	0
NA	2	0	0	0	0	0	0
NA	1	0	0	1	0	0	0
NA	1	0	0	0	0	0	0
NA	2	0	0	2	0	0	0
NA	1	1	0	0	0	0	0
NA	1	1	0	0	0	0	0
NA	1	0	0	0	0	0	1
auratus	2	0	0	0	0	0	0
NA	2	0	0	0	0	0	0
destructans	113	0	0	0	0	0	0
NA	23	0	0	12	0	0	1
NA	7	0	0	3	0	1	0
NA	1	1	0	0	0	0	0

*bovicornutus** 99%	1	1	0	0	0	0	0
hansenii	87	0	1	19	18	21	0
sp. 1	56	12	2	0	22	5	0
sp. 2	1	0	0	1	0	0	0
sp. 3	49	0	24	5	1	9	0
sp. 4	3	0	0	1	0	0	2
sp. 5	4	0	0	4	0	0	0
phyllophila	1	0	0	0	0	0	0
lactis	1	0	0	1	0	0	0
NA	1	1	0	0	0	0	0
palmioleophila	1	0	0	0	0	0	0
apis	1	0	0	1	0	0	0
NA	1	1	0	0	0	0	0
leptobactrum	1	1	0	0	0	0	0
leptobactrum	2	0	0	0	0	0	0
leptobactrum	1	0	0	0	0	0	0
chlamydosporia	1	0	0	1	0	0	0
muscarium	1	0	0	0	1	0	0
NA	3	0	0	0	0	0	0
NA	1	0	0	0	0	0	0
NA	1	0	0	0	0	0	0
lanosoniveum	1	0	0	0	0	0	0
NA	2	2	0	0	0	0	0
NA	1	0	0	1	0	0	0
stellatum	1	1	0	0	0	0	0
NA	1	0	0	0	0	0	0
NA	1	0	0	0	0	0	0
caviariforme	5	0	0	5	0	0	0
stemonitis	1	0	0	0	0	0	0
aggregata	1	0	0	0	0	1	0
simplex	5	0	1	0	0	0	0
brumptii	1	1	0	0	0	0	0
humicola	2	0	0	0	1	0	0
inflata	1	0	0	0	1	0	0
mexicana	3	0	0	1	0	0	0
hypophloia	1	0	0	1	0	0	0
atrogriseum	1	0	0	1	0	0	0
NA	1	0	0	1	0	0	0
NA	1	1	0	0	0	0	0
NA	1	0	0	0	1	0	0
aeria	1	1	0	0	0	0	0
pinicola	2	2	0	0	0	0	0

slooffiae	3	3	0	0	0	0	0
coprosmae	1	1	0	0	0	0	0
hasegawianum	1	1	0	0	0	0	0
phylloplanum	1	0	1	0	0	0	0
vespertilionis	59	0	0	0	0	9	1
NA	1	0	0	1	0	0	0
NA	18	0	0	17	0	0	0
macerans	2	0	0	0	1	0	0
pullulans	2	0	0	1	0	0	0
pyricola	1	0	0	0	1	0	0
magnum	2	1	0	0	0	0	0
takashimae	1	0	0	0	0	0	0
luteola	1	0	0	1	0	0	0
oryzae	1	0	0	1	0	0	0
carnescens	4	0	0	4	0	0	0
tephrensis	2	0	1	1	0	0	0
victoriae	3	0	0	2	0	0	0
heveanensis	1	1	0	0	0	0	0
pini	1	1	0	0	0	0	0
flavescens	1	0	0	0	0	0	0
NA	1	1	0	0	0	0	0
dulcitum	5	0	0	0	0	0	0
guehoae	1	0	0	0	0	0	0
moniliiforme	49	0	0	0	13	15	0
NA	1	0	0	0	0	0	0
NA	2	0	0	0	0	2	0
fragicola	5	0	0	0	0	0	0
alpina	1	1	0	0	0	0	0
beljakovae	1	0	0	0	0	0	0
clonocystis	1	1	0	0	0	0	0
dichotoma	11	0	0	0	0	0	0
NA	1	0	0	0	0	0	0
NA	1	0	0	0	0	0	0
NA	3	0	0	0	0	0	0
NA	5	5	0	0	0	0	0
parvispora	2	0	0	0	0	0	0
parvispora	2	0	0	0	0	0	0
pulchrum	1	0	0	0	0	0	0
NA	1	0	0	0	1	0	0
NA	7	7	0	0	0	0	0

Table 2.S1 continued

MYLU	MYLUr	MYSE	MYSO	PESU
0	1	1	12	0
0	0	0	0	0
0	0	0	0	0
0	1	0	0	0
0	0	0	0	0
0	0	1	0	0
0	1	0	1	1
0	0	0	0	0
0	0	0	0	0
0	0	0	0	1
0	0	1	0	0
3	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
1	1	2	6	0
0	0	0	0	0
1	0	0	6	0
0	0	0	0	0
0	0	0	0	0
1	0	0	1	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	1
1	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	1
0	0	0	0	0
0	0	0	0	0
0	0	0	0	1
0	0	3	0	0
0	0	0	0	0
0	0	0	0	1

0	0	1	0	0
0	0	1	0	1
0	0	0	0	0
0	0	0	0	1
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	1	0	1	0
0	1	1	0	0
4	57	2	11	39
3	1	4	1	1
1	0	2	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	28	0
2	7	2	0	4
0	0	0	0	0
0	2	0	7	1
0	0	0	0	0
0	0	0	0	0
0	1	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	1
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	2	0	0
0	0	1	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	1	2
0	0	1	0	0
0	0	0	0	1
0	0	1	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	1	0	0
1	0	0	0	0
0	0	0	0	0
0	0	1	0	0

0	0	0	0	0
0	4	0	0	0
0	0	0	0	0
0	0	1	0	0
0	0	0	0	0
0	0	2	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
20	5	10	14	0
0	0	0	0	0
0	0	1	0	0
0	0	0	0	1
0	0	0	0	1
0	0	0	0	0
1	0	0	0	0
0	1	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
1	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	1
0	0	0	0	0
0	2	0	2	1
0	0	0	1	0
0	1	2	18	0
0	0	1	0	0
0	0	0	0	0
0	0	0	5	0
0	0	0	0	0
0	0	0	0	1
0	0	0	0	0

3	7	1	0	0
1	0	0	0	0
0	0	1	0	0
0	0	3	0	0
0	0	0	0	0
1	0	1	0	0
0	0	2	0	0
0	1	0	0	0
0	0	0	0	0
0	0	0	0	0

Table 2.S2: The top six best zero-inflated models for four different response variables (Shannon Index and abundance, both for all fungi and yeast only) based on Akaike information criterion (AIC). Multiple combinations of variables were tested to determine each variable's relative influence on each response variable. The difference in AIC between the best model (dAIC=0) and next best models are listed. Models with dAIC < 4 are considered identical. Gaussian distributions were the best in all cases. Fungal and yeast abundance are based on counts of colony forming units. Group= white-nose syndrome (WNS)-susceptibility group.

dAIC	df	Model
		Shannon Diversity Index, all fungi
0	13	~ Bat Species + (1|Site)
4.4	7	~ Group + (1|Bat Species) + (1|Site)
5.5	9	~ Group + (1|Site)
9.5	11	~ Group + (1|Day of Year) + (1|Site)
28.8	13	~ Group + State
32.6	25	~ Bat Species + Day of Year + Days to Plate
		Shannon Diversity Index, yeast only
0	23	~ Bat Species + (1|Month)
35.4	11	~ Group + (1|Bat Species) + (1|State)
63	7	~ Group + Day of Year + Days to Plate
64.1	9	~ Group + Day of Year + Days to Plate + Month
64.2	12	~ Bat Species
64.7	13	~ Bat Species + Days to Plate
		Fungal Abundance
0	23	~ Bat Species + (1|Day of Year)
2.2	23	~ Bat Species + (1|State)
3.4	25	~ Bat Species + (1|Day of Year) + (1|Site)
3.8	23	~ Bat Species + (1|Site)
10.1	25	~ Bat Species + Month

17.8	23	~ Bat Species + (1|Days to Plate)
		Yeast Abundance
0	23	~ Bat Species + (1|Day of Year)
1.7	23	~ Bat Species + (1|State)
2.5	25	~ Bat Species + (1|Day of Year) + (1|Site)
2.9	23	~ Bat Species + (1|Site)
8	27	~ Bat Species + (1|Day of Year) + (1|Month) + (1|Days to Plate)
9.3	25	~ Bat Species + Month

Table 2.S3: Results of the best model for each response variable (Shannon Index and abundance, both for all fungi and yeast only, Table S2). P-values in bold are considered significant (p-value < 0.05). SE=standard error.

Response Variable	Shannon Diversity - all fungi			Shannon Diversity - yeast only			Fungal Abundance			Yeast Abundance		
Model	~ Bat Species + (1|Site)			~ Bat Species + (1|Month)			~ Bat Species + (1|Day of Year)			~ Bat Species + (1|Day of Year)		
Bat Species	Estimate	SE	p-value	Estimate	SE	P-value	Estimate	SE	P-value	Estimate	SE	P-value
Corynorhinus rafinesquii	0.34	0.09	**0.0003**	0.11	0.03	**0.0012**	1.44	0.35	**4.45E-05**	1.56	0.35	**8.95E-06**
C. townsendii virginianus	0.03	0.13	**0.0158**	0.03	0.05	0.0978	1.81	0.4	0.362	1.74	0.4	0.6617
Eptesicus fuscus	0.63	0.13	**0.0245**	0.65	0.05	**2.00E-16**	2.22	0.41	0.056	2.18	0.42	0.1385
Myotis austroriparius	0.47	0.12	0.2905	0.83	0.06	**2.00E-16**	3.06	0.4	**5.88E-05**	3.16	0.4	**7.26E-05**
M. grisescens	0.7	0.13	**0.0053**	0.8	0.05	**2.00E-16**	3.15	0.41	**2.94E-05**	3.12	0.41	**0.0001**
M. leibii	0.36	0.19	0.9177	0.21	0.09	0.2947	1.38	0.5	0.898	1.33	0.51	0.6513
M. lucifugus	0.19	0.12	0.2217	0.05	0.04	0.173	1.55	0.41	0.788	1.92	0.43	0.3984
M. septentrionalis	0.19	0.11	0.1907	0.03	0.04	0.0764	1.63	0.4	0.633	1.58	0.41	0.9619
M. sodalis	0.63	0.12	**0.0154**	0.71	0.05	**2.00E-16**	3.12	0.4	**2.42E-05**	3.09	0.4	**0.0001**
Perimyotis subflavus	0.03	0.11	**0.0034**	0.02	0.04	**0.0132**	1.07	0.43	0.39	1.26	0.44	0.4997

Table 2.S4: Bat species from which *Pseudogymnoascus destructans* (*Pd*) was cultured. Sites are listed by state followed by the site number. The mean number of colony forming units (CFU) for all fungi totaled over the three agar types used ± the standard deviation is given with the number of *Pd* colonies included in one column and excluded in another. *Pd* was not cultured from *Corynorhinus rafinesquii, C. townsendii, Eptesicus fuscus, M. austroriparius, M. grisescens*, or *M. leibii* during this study. NA = not applicable. MYLU = *M. lucifugus*, MYLUr = *M. lucifugus* from white-nose syndrome (WNS)-resistant colonies New York, MYSE = *M. septentrionalis*, MYSO = *M. sodalis*, PESU = *Perimyotis subflavus*, WI = Wisconsin, KY = Kentucky, NY = New York, AL = Alabama, MO = Missouri, OK = Oklahoma, WV = West Virginia.

Bat Species	Site	Mean CFU with *Pd*	Mean CFU without *Pd*	# of bats	% *Pd* positive
MYLU	WI1	NA	42.2 ± 78.2	6	0
MYLU	WI2	NA	0	1	0
MYLU	WI3	23 ± 34.7	22.2 ± 38.8	25	12
MYLU	WI9	830.5 ± 385.4	1.7 ± 2.9	10	100
MYLU	KY1	100	0	1	100
MYLU	**all sites**			**43**	**32.6**
MYLUr	NY2	44.9 ± 39.0	2.1 ± 4.0	30	100
MYLUr	NY1	54.8 ± 40.7	3.4 ± 9.2	31	83.9
MYLUr	**all sites**			**61**	**91.8**
MYSE	WI2	NA	14.6 ± 27.6	16	0
MYSE	WI4	28.5 ± 2.1	21 ± 18.4	3	66.7
MYSE	WI5	NA	5.3 ± 8.5	6	0
MYSE	WI6	NA	32.2 ± 44.2	6	0
MYSE	WI8	NA	3.3 ± 4.9	8	0
MYSE	**all sites**			**39**	**5.1**
MYSO	AL1	NA	131.5 ± 4.9	2	0
MYSO	MO1	387.5 ± 195.1	386.5 ± 194.9	11	27.3
MYSO	KY1	161.3 ± 106.1	135.9 ± 105.5	15	80
MYSO	**all sites**			**28**	**53.6**
PESU	MO1	114.7 ± 191.6	2.7 ± 4.4	6	100
PESU	KY1	56.6 ± 75.3	0.1 ± 0.4	30	100
PESU	AL1	3 ± 2.8	0 ± 0	5	40
PESU	AL2	NA	2.7 ± 3.1	3	0
PESU	AL3	NA	1.6 ± 3.6	5	0
PESU	OK1	NA	7.2 ± 24.0	17	0
PESU	WI2	NA	0 ± 0	10	0
PESU	WI7	NA	0	1	0
PESU	WI8	NA	0	1	0
PESU	WV2	52	0	1	100
PESU	**all sites**			**79**	**49.4**

Table 2.S5: Primers and cycling conditions for phylogenetic analyses.

Gene	Taxa	Primers (5' → 3')	Primer Use	Reference	Cycling Conditions
ITS	*Cutaneotrichosporon*	GGAAGTAAAAGTCGTAACAAGG	amplification/ sequencing	(White *et al.*, 1990)	98C for 2 min; 40 cycles of 98C for 10 sec, 50C for 30 sec,
	Debaryomyces	TCCTCCGCTTATTGATATGC	amplification/ sequencing		

	Leucosporidium				72C for 1 min; 72C for 7 min
COX2	*Debaryomyces*	GGTATWCAKGAATTATAYGA	amplification/ sequencing	(Tsui *et al*., 2007)	95C for 5 min; 45 cycles of 95C for 30 sec, 46C for 30 sec, 72C for 45 sec; 72C for 7 min
		CASATTCWARTTTKGTWGGCAT	amplification/ sequencing		
ACT1	*Debaryomyces*	ATTGATAACGGTTCCGGTATGTG	amplification/ sequencing	(Daniel and Meyer, 2003)	94C for 5 min; 45 cycles of 94C for 30 sec, 45C for 30 sec, 72C for 1 min; 72C for 10 min
		TCGTCGTATTCTTGCTTTGAGATCCAC	amplification/ sequencing		
D1-D2	*Cutaneotrichosporon*	GCATATCAATAAGCGGAGGAAAAG	amplification/ sequencing	(Kurtzman and Robnett, 1997)	94C for 5 min; 30 cycles of 94C for 45 sec, 51C for 1 min, 72C for 3 min; 72C for 10 min
	Debaryomyces	GGTCCGTGTTTCAAGACGG	amplification/ sequencing		
	Leucosporidium				
RPB2	*Cutaneotrichosporon*	GAYGAYMGWGATCAYTTYGG	amplification/ sequencing	(Liu *et al*., 1999)	94C for 5 min; 45 cycles of 94C for 30 sec, 45C for 30 sec, 72C for 1 min; 72C for 7 min
	Debaryomyces	CCCATRGCTTGYTTRCCCAT	amplification/ sequencing		
TEF1	*Cutaneotrichosporon*	GCYCCYGGHCAYCGTGAYTTYAT	amplification/ sequencing	(Rehner and Buckley, 2005)	94C for 2 min; 47 cycles of 94C for 1 min, 53C for 1 min, 72C for 1 min and 40 s; 72C for 10 min
		ATGACACCRACRGCRACRGTYTG	amplification/ sequencing		
		CAGGAYGTNTACAAGATYGGTGG	sequencing		
		ACHGTRCCRATACCACCRATCTT	sequencing		

Table 2.S6: GenBank accession numbers of sequenced loci and models used for phylogenetic analyses.

Isolate	**Taxon**	**GenBank Accession numbers: ITS**
44797-84 I1-DTM	*Debaryomyces* sp. 1	MK275220
44797-84 I2-LNA	*Debaryomyces* sp. 1	MK275221
24686-1 I2-SD	*Debaryomyces* sp. 2	MK275218
24716-19 I2-SD	*Debaryomyces* sp. 2	MK275208
24729-12 I2-SD	*Debaryomyces* sp. 2	MK275219
24738-13 I1-SD	*Debaryomyces* sp. 2	MK275209
44768-15 I2-SD	*Debaryomyces* sp. 2	MK275210
44769-25 I1-SD	*Debaryomyces* sp. 2	MK275211
44797-78 I2-SD	*Debaryomyces* sp. 2	MK275212
44797-136 I2-SD	*Debaryomyces* sp. 2	MK275213
44797-166 I2-SD	*Debaryomyces* sp. 2	MK275214
44797-167 I1-SD	*Debaryomyces* sp. 2	MK275215
44797-170 I1-SD	*Debaryomyces* sp. 2	MK275216
44797-144 I4-SD	*Debaryomyces* sp. 2	MK275217
24728-13 I3-SD	*Debaryomyces* sp. 3	MK275223

24729-11 I1-SD	*Debaryomyces* sp. 3	MK275225
44767-23 I3-SD	*Debaryomyces* sp. 3	MK275226
44768-29 I2-SD	*Debaryomyces* sp. 3	MK275224
44797-66 I3-SD	*Debaryomyces* sp. 3	MK275227
45704-144 I4-SD	*Debaryomyces* sp. 4	MK275222
44797-87 I1-DTM	*Debaryomyces* sp. 5	MK275205
44797-91 I1-DTM	*Debaryomyces* sp. 5	MK275206
44797-97 I2-DTM	*Debaryomyces* sp. 5	MK275207
24686-2 I1-SD	*Debaryomyces hansenii*	MK275228
44767-27 I1-SD	*Debaryomyces hansenii*	MK275231
44797-62 I2-SD	*Debaryomyces hansenii*	MK275232
44797-133 I1-SD	*Debaryomyces hansenii*	MK275233
44797-190 I1-SD	*Debaryomyces hansenii*	MK275229
44797-193 I2-SD	*Debaryomyces hansenii*	MK275230
NRRL Y-7425/CBS 5167 (type strain)	*Debaryomyces coudertii*	AB054018
NRRL Y-17914/CBS 789 (type strain)	*Debaryomyces fabryi*	AJ586530
NRRL Y-7426/CBS 767 (type strain)	*Debaryomyces hansenii* var. *hansenii*	AJ586526
NRRL Y-2171/CBS 1958 (type strain)	*Debaryomyces maramus*	AJ586525
NRRL Y-7108/CBS 5921 (type strain)	*Debaryomyces nepalensis*	AB053099
NRRL Y-27369/CBS 8450 (type strain)	*Debaryomyces prosopidis*	JN942657
NRRL Y-48723/CBS 11845 (type strain)	*Debaryomyces psychrosporus*	HM769277
NRRL Y-6670/CBS 2934 (type strain)	*Debaryomyces robertsiae*	AJ586522
NRRL Y-7859/CBS 792 (type strain)	*Debaryomyces subglobosus*	FN675240
NRRL Y-17354/CBS 7056 (type strain)	*Debaryomyces udenii*	AB054098
NRRL YB-4275/CBS 2285 (type strain)	*Priceomyces carsonii*	AJ586521
Model used for Phylogenetic Analysis		GTR+G
Number of Characters Used in Analysis		547
24728-13 I2SD	*Leucosporidium* sp.	MK271678
44797-122 I2SD	*Leucosporidium* sp.	MK271679
44797-083 I3-SD	*Leucosporidium* sp.	MK271680
44797-086 I1-SD	*Leucosporidium* sp.	MK271681
44797-091 I2-SD	*Leucosporidium* sp.	MK271682
45704-142 I3-SD	*Leucosporidium* sp.	MK271683
CBS 7287 (type strain)	*Leucosporidium fellii*	AF444508
CBS 7226 (type strain)	*Leucosporidium intermedium*	AF444564
PYCC 6879 (type strain)	*Leucosporidium krtinense*	KU187882
CBS 5930 (type strain)	*Leucosporidium scottii*	AF444495
CBS 8621 (type strain)	*Leucosporidium yakuticum*	AY212989
CBS 8620 (type strain)	*Leucosporidium creatinivorum*	AF444629
CBS 12734 (type strain)	*Leucosporidium escuderoi*	NR_155310
CBS 11562 (type strain)	*Leucosporidium drummii*	KY104017
CBS 6254 (type strain)	*Leucosporidium fragarium*	AF444530

PYCC 5759 (type strain)	*Leucosporidium golubevii*	AY212987
CBS 6921 (type strain)	*Leucosporidium muscorum*	AF444527
CBS 4240 (type strain)	*Sampaiozyma ingeniosa*	AF444534
Model used for Phylogenetic Analysis		K2+G
Number of Characters Used in Analysis		492
44797-120 I3-SD	*Cutaneotrichosporon* sp. 1	MK271642
44767-23 I2-SD	*Cutaneotrichosporon* sp. 2	MK271643
44767-31 I2-SD	*Cutaneotrichosporon* sp. 2	MK271644
44767-40 I2-SD	*Cutaneotrichosporon moniliiforme*	MK271645
44797-65 I2-SD	*Cutaneotrichosporon moniliiforme*	MK271646
44797-65 I2-DTM	*Cutaneotrichosporon moniliiforme*	MK271647
44797-127 I1-SD	*Cutaneotrichosporon moniliiforme*	MK271648
44797-142 I2-SD	*Cutaneotrichosporon moniliiforme*	MK271649
44797-153 I1-DTM	*Cutaneotrichosporon moniliiforme*	MK271650
CBS 2466 (type strain)	*Cutaneotrichosporon cutaneum*	AF444325
CBS 6864 (type strain)	*Cutaneotrichosporon jirovecii*	AF444437
ATCC 20509 (type strain)	*Cutaneotrichosporon oleaginosus*	HM802135
CBS 8370 (type strain)	*Cutaneotrichosporon smithiae*	AF444397
CBS 9546 (type strain)	*Cutaneotrichosporon terricola*	AB031517
CBS 2467 (type strain)	*Cutaneotrichosporon moniliiforme*	AF444415
CBS 2043 (type strain)	*Cutaneotrichosporon dermatis*	AY143557
CBS 7625 (type strain)	*Cutaneotrichosporon mucoides*	AF444423
CBS 1896 (type strain)	*Cutaneotrichosporon debeurmannianum*	AY143556
CBS 10441 (type strain)	*Cutaneotrichosporon arboriformis*	AB260936
CBS 8902 (type strain)	*Cutaneotrichosporon haglerorum*	AY787857
CBS 8521 (type strain)	*Cutaneotrichosporon guehoae*	AF410476
CBS 5123 (type strain)	*Cutaneotrichosporon daszewskae*	AB035580
CBS 11948 (type strain)	*Cutaneotrichosporon cyanovorans*	JF680900
CBS 570 (type strain)	*Cutaneotrichosporon curvatus*	AF410467
CBS 11177 (type strain)	*Haglerozyma chiarellii*	GQ338074
CBS 8257 (type strain)	*Apotrichum dulcitum*	AF444428
CBS 8898 (type strain)	*Vanrija fragicola*	AB035588
Model used for Phylogenetic Analysis		GTR+G
Number of Characters Used in Analysis		407

Table 2.S6 continued

GenBank Accession Numbers				
D1/D2	***ACT1***	***RPB2***	***COX2***	***TEF1***
MK273536	MK300125	MK300189	MK300157	-
MK273537	MK300126	MK300190	MK300158	-
MK273534	MK300123	MK300187	MK300155	-

MK273524	MK300113	MK300177	MK300145	-
MK273535	MK300124	MK300188	MK300156	-
MK273525	MK300114	MK300178	MK300146	-
MK273526	MK300115	MK300179	MK300147	-
MK273527	MK300116	MK300180	MK300148	-
MK273528	MK300117	MK300181	MK300149	-
MK273529	MK300118	MK300182	MK300150	-
MK273530	MK300119	MK300183	MK300151	-
MK273531	MK300120	MK300184	MK300152	-
MK273532	MK300121	MK300185	MK300153	-
MK273533	MK300122	MK300186	MK300154	-
MK273539	MK300128	MK300192	MK300160	-
MK273541	MK300130	MK300194	MK300162	-
MK273542	MK300131	MK300195	MK300163	-
MK273540	MK300129	MK300193	MK300161	-
MK273543	MK300132	MK300196	MK300164	-
MK273538	MK300127	MK300191	MK300159	-
MK273521	MK300110	MK300174	MK300142	-
MK273522	MK300111	MK300175	MK300143	-
MK273523	MK300112	MK300176	MK300144	-
MK273544	MK300133	MK300197	MK300165	-
MK273547	MK300136	MK300200	MK300168	-
MK273548	MK300137	MK300201	MK300169	-
MK273549	MK300138	MK300202	MK300170	-
MK273545	MK300134	MK300198	MK300166	-
MK273546	MK300135	MK300199	MK300167	-
U45846	AJ867051	MK300203	AM991995	-
EU816296	AJ508504	EF599461	EF599378	-
U45808	AJ508505	AY497624	EF599377	-
JN940502	AJ606311	MK300204	MK300171	-
U45839	AJ867054	MK300205	AM991996	-
JN940510	AM992977	MK300206	AM991990	-
HM769275	MK300139	MK300207	MK300172	-
U45805	AJ867053	MK300208	AM991994	-
FN675239	MK300140	MK300209	MK300173	-
U45844	MK300141	MK300210	AM991997	-
U45743	AJ508503	MK300211	EF599380	-
K2+G	K2+G	GTR+G+I	GTR+G	
530	876	960	579	
MK271684	-	-	-	-
MK271685	-	-	-	-
MK271686	-	-	-	-
MK271687	-	-	-	-

MK271688	-	-	-	-
MK271689	-	-	-	-
AF189907	-	-	-	-
AF189889	-	-	-	-
KU187886	-	-	-	-
AY213000	-	-	-	-
AY213001	-	-	-	-
AF189925	-	-	-	-
NG_060273	-	-	-	-
NG_057823	-	-	-	-
AF070428	-	-	-	-
AY212997	-	-	-	-
AF070433	-	-	-	-
AF189934	-	-	-	-
K2+G 565				
MK271651	-	MK300092	-	MK300101
MK271652	-	MK300093	-	MK300102
MK271653	-	MK300094	-	MK300103
MK271654	-	MK300095	-	MK300104
MK271655	-	MK300098	-	MK300106
MK271656		MK300096		MK300105
MK271657	-	MK300099	-	MK300107
MK271658	-	MK300097	-	MK300109
MK271659	-	MK300100	-	MK300108
AF075483	-	KF036961	-	KF037221
AF105398	-	KF036974	-	KF037234
HM802132	-	NW_017264987	-	NW_017264999
AF444706	-	KF036987	-	KF037244
AB086382	-	KF036989	-	-
AF105392	-	KF036979	-	KF037238
AY143555	-	KF036964	-	KF037224
AF075515	-	KF036981	-	KF037240
AY143554	-	KF036962	-	KF037222
AB260936	-	KF036760	-	KF037032
AF407276	-	KF036787	-	KF037059
AF105401	-	GCA_001600415	-	GCA_001600415
AB126588	-	KF036773	-	KF037046
JF680899	-	GCA_002335625	-	GCA_002335625
AF189834	-	KF036771	-	KF037044
EU030272	-	KF036959	-	KF037219
AF075517	-	KF036967	-	KF037227
AB126585	-	KF036782	-	KF037054

K2+G	GTR+G+I	GTR+G
595	880	870

ATCC = American Type Culture Collection
NRRL = Agricultural Research Service (ARS) Culture Collection
CBS = CBS KNAW Culture Collection
GTR+G = general time reversible model with a gamma distribution
GTR+G+I = general time reversible model with a gamma distribution and invariant sites
K2+G = Kimura 2-parameter model with gamma distribution

Table 2.S7: The number of fungal operational taxonomic units (OTUs) per bat comparing culture-independent (CI) to culture-dependent (CD) results. CFU = colony forming units

Bat Species	Site	# OTUs by CI	# OTUs by CD	# of shared OTUs	% of sequences generated by CI detected by CD	% of CFU generated by CD detected by CI
Myotis austroriparius	Alabama #2	6	4	3	66.6	99.5
M. austroriparius	Alabama #2	9	2	1	49.6	100
M. septentrionalis	Wisconsin #6	17	5	5	54.5	98.6
M. septentrionalis	Wisconsin #6	12	4	3	74.3	50
M. septentrionalis	Wisconsin #6	8	3	2	53.2	77.8
M. sodalis	Missouri #1	19	5	4	73	98.2
M. sodalis	Missouri #1	9	5	2	83.9	95.3

Table 2.S8: Fungal taxa detected from the wing surface of bats in the Eastern United States using next-generation sequencing. Numbers indicate the number of reads obtained for each fungal operational taxonomic units (OTU). Fungal taxa indicated by * were identified using NCBI BLAST function; all other taxa were identified using UNITE. “NA” indicates the OTU could not be identified with available databases. Numbers highlighted in green were also detected using culture-dependent methods. MYSO = *Myotis sodalis*, MYSE = *M. septentrionalis*, and MYAU = *M. austroriparius*.

Phylum	**Class**	**Order**	**Family**	**Genus**
Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Epicoccum* 100%*
Ascomycota	Eurotiomycetes	Chaetothyriales	Trichomeriaceae	NA
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	*Cistella*
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*

Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*
Ascomycota	Leotiomycetes	Helotiales	Myxotrichaceae	*Oidiodendron*
Ascomycota	Leotiomycetes*	Helotiales*	Myxotrichaceae*	NA
Ascomycota	Leotiomycetes*	Helotiales*	NA	NA
Ascomycota	Leotiomycetes	Helotiales	NA	NA
Ascomycota	Leotiomycetes	Helotiales	Vibrisseaceae	*Phialocephala*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudeurotium* 100%*
Ascomycota	Leotiomycetes	Thelebolales*	Pseudeurotiaceae*	*Pseudeurotium* 100%*
Ascomycota	Leotiomycetes	Thelebolales*	Pseudeurotiaceae*	*Pseudeurotium* 100%*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudogymnoascus**
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudogymnoascus* 100%*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudogymnoascus* 100%*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudogymnoascus* 100%*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudogymnoascus* 100%*
Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	*Pseudogymnoascus* 100%*
Ascomycota	Pezizomycetes	Pezizales	Pezizaceae	*Peziza*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetales	*Incertae sedis*	*Candida*
Ascomycota	Saccharomycetes	Saccharomycetales	Trichomonascaceae	*Blastobotrys*
Ascomycota	Sordariomycetes	Chaetosphaeriales	Chaetosphaeriaceae	*Chloridium*
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Verticillium*
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Verticillium*
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Verticillium*
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Verticillium*
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Verticillium*
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Hypomyces*
Ascomycota	Sordariomycetes	Microascales	Microascaceae	*Acaulium*
Ascomycota	Sordariomycetes	Sordariales	Cephalothecaceae	*Cryptendoxyla*

Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	NA
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	NA
Ascomycota	Sordariomycetes	Xylariales	Sporocadaceae	*Pestalotiopsis*
Ascomycota	Sordariomycetes	Xylariales	Sporocadaceae	*Pestalotiopsis*
Ascomycota*	NA	NA	NA	NA
Basidiomycota	Agaricomycetes	Amylocorticiales	Amylocorticiaceae	*Amylocorticiellum*
Basidiomycota	Agaricomycetes	Polyporales	Ganodermataceae	*Ganoderma* 99%*
Basidiomycota*	Malasseziomycetes*	Malasseziales*	Malasseziaceae*	*Malassezia**
Basidiomycota	Malasseziomycetes*	Malasseziales*	NA	NA
Basidiomycota	Malasseziomycetes*	Malasseziales*	NA	NA
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae*	NA
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae*	NA
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae*	NA
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Apiotrichum*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Vanrija*
Chytridiomycota*	Chytridiomycetes*	NA	NA	NA
Chytridiomycota*	Chytridiomycetes*	NA	NA	NA
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*
Mortierellomycota*	Mortierellomycetes*	Mortierellales*	Mortierellaceae*	*Mortierella* 95%*
Mucoromycota	Mucoromycetes	Mucorales	Mucoraceae	*Mucor*

Table 2.S8 continued

Species	**MYSO**	**MYSO**	**MYSE**	**MYSE**	**MYSE**	**MYSE**	**MYAU**	**MYAU**
NA	0	209	0	0	0	0	3543	495
NA	0	0	0	0	0	0	0	161
NA	0	54	0	0	0	0	0	0
NA	0	0	0	0	0	0	0	2078
NA	0	0	398	0	115	0	0	0
NA	0	113	0	0	0	0	0	0
acuum	0	0	2138	0	0	0	0	0
NA	0	0	345	0	191	0	0	0
NA	0	0	0	137	0	0	0	0
NA	0	0	0	75	0	0	0	0

NA	0	50	0	0	0	0	0	0
NA	0	19	0	0	0	0	0	0
truncatum 100%*	0	0	0	161	0	0	0	0
NA	0	0	0	165	0	0	0	0
NA	0	0	0	0	0	0	0	1300
NA	0	0	143	0	0	0	0	0
NA	0	24	0	0	0	0	0	0
NA	0	0	364	0	0	0	0	0
NA	0	0	471	398	0	0	0	0
NA	0	0	748	0	0	0	0	0
roseus 100%*	0	0	166	0	0	0	0	0
NA	0	0	5892	538	262	0	0	0
NA	0	0	609	0	0	0	0	0
NA	0	0	327	0	0	0	0	0
NA	0	91	0	0	0	0	0	0
NA	35	40	0	0	0	0	0	0
NA	0	10	0	0	0	0	0	0
hansenii	1850	6963	0	0	0	0	1931	1010
hansenii	0	0	0	0	0	0	5187	3603
hansenii	729	3748	0	0	0	0	0	0
hansenii	0	0	0	0	0	0	171	137
hansenii	0	217	0	0	0	0	0	0
hansenii	0	215	0	0	0	0	0	0
hansenii	0	0	0	0	0	0	162	0
hansenii	0	120	0	0	0	0	0	0
NA	0	0	0	239	0	0	0	0
buckinghamii	0	0	824	0	0	0	0	0
NA	0	0	0	0	0	0	301	0
leptobactrum	0	0	195	0	0	0	0	0
NA	0	0	0	0	0	0	280	0
NA	0	0	0	0	0	0	0	83
NA	0	0	0	0	77	0	0	0
NA	0	0	0	37	0	0	0	0
NA	0	0	0	76	0	0	0	0
caviariforme	0	0	102	0	0	0	0	0
hypophloia	0	0	0	265	0	0	0	0
NA	0	0	0	0	0	0	0	126
NA	0	35	0	0	0	0	0	0
NA	0	0	0	0	0	0	771	0
NA	0	0	0	0	0	0	311	0
NA	0	0	0	36	0	0	0	0
molle	0	0	0	0	240	0	0	0

NA	0	0	161	0	0	0	0	0
*vespertilionis**	110	1611	0	0	0	0	0	0
NA	80	797	0	0	0	0	0	576
NA	0	208	0	0	0	0	0	0
NA	0	0	179	0	409	0	0	0
NA	0	0	0	0	151	0	0	0
NA	0	0	0	0	70	0	0	0
scarabaeorum	13	338	0	0	0	0	0	0
guehoae	0	97	0	0	0	0	0	0
moniliiforme	40	175	0	0	0	0	2921	0
NA	0	0	0	5013	0	0	0	0
NA	0	0	0	179	0	0	0	0
NA	0	0	0	146	0	0	0	0
fragicola	188	990	0	0	0	0	0	0
NA	0	3	0	0	0	0	0	0
NA	0	0	0	0	0	0	0	15
hyalina	0	0	0	0	527	0	0	0
macrocystis	0	0	40	0	0	0	0	0
NA	0	0	400	0	0	0	0	0
parvispora	69	1625	0	667	132	0	0	0
NA	0	0	183	0	0	0	0	0
flavus	8	278	0	0	0	0	0	0

Chapter 3 Supplemental Data

Table 3.S1: Yeast taxa cultured from the wing surface of bats in western North America. Aside from total sample size for each species (row 2), numbers indicate the number of individual bats each fungal operational taxonomic unit (OTU) was cultured from. In most cases multiple OTUs were cultured from an individual bat (thus, sums may exceed the sample size listed in row 2). Fungal taxa indicated by * were identified using NCBI BLAST function, all other taxa were identified using UNITE. "NA" indicates the OTU could not be identified with available databases.

Phylum	Class	Order	Family	Genus
Total sample size for each species				
Ascomycota	Arthoniomycetes	Lichenostigmatales	Phaeococcomycetaceae	*Phaeococcomyces*
Ascomycota	Arthoniomycetes	Lichenostigmatales	Phaeococcomycetaceae	*Phaeococcomyces*
Ascomycota	Dothideomycetes	Dothideales	Aureobasidiaceae	*Aureobasidium*

Ascomycota	Dothideomycetes	Dothideales	Aureobasidiaceae	*Aureobasidium*
Ascomycota	Dothideomycetes	Dothideales	Aureobasidiaceae*	*Aureobasidium* *98%
Ascomycota	Dothideomycetes	Dothideales	Dothideaceae	*Dothidea*
Ascomycota	Dothideomycetes	Dothideales	Dothideaceae	*Endoconidioma*
Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	*Dothiora*
Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	*Hormonema*
Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	*Hormonema*
Ascomycota	Dothideomycetes	Myriangiales	NA	NA
Ascomycota	Dothideomycetes	Myriangiales	NA	NA
Ascomycota	Dothideomycetes	Mytilinidiales	Mytilinidiales_fam_Incertae_sedis	*Halokirschsteiniotheli a*
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Epicoccum* *
Ascomycota	Dothideomycetes	Pleosporales	Melanommataceae	*Alpinaria*
Ascomycota	Dothideomycetes *	Dothideales *	Dothideomycetidae *	*Zalaria* *100%
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Exophiala*
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Exophiala*
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Phialophora*
Ascomycota	Eurotiomycetes	Chaetothyriales	Trichomeriaceae	*Knufia*
Ascomycota	Eurotiomycetes	Dothideales	Cyphellophoraceae	*Cyphellophora*
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Talaromyces*
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Talaromyces*
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	*Collophora*
Ascomycota	Leotiomycetes	Helotiales	NA	NA
Ascomycota	Leotiomycetes	Helotiales	NA	NA
Ascomycota	Leotiomycetes	Helotiales	NA	NA
Ascomycota	Leotiomycetes	Helotiales	Vibrisseaceae	*Phialocephala*
Ascomycota	Leotiomycetes	Thelebolales	Thelebolaceae	*Thelebolus*
Ascomycota	NA	NA	NA	NA
Ascomycota	Saccharomycetes	Saccharomycetale s	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetale s	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetale s	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetale s	Debaryomycetaceae	*Debaryomyces*
Ascomycota	Saccharomycetes	Saccharomycetale s	Metschnikowiaceae	*Metschnikowia*
Ascomycota	Saccharomycetes	Saccharomycetale s	NA	NA
Ascomycota	Saccharomycetes	Saccharomycetale s	NA	NA
Ascomycota	Saccharomycetes	Saccharomycetale s	Pichiaceae	*Nakazawaea*
Ascomycota	Saccharomycetes	Saccharomycetale s	Saccharomycetales_fam_Incertae_se dis	*Candida*
Ascomycota	Saccharomycetes	Saccharomycetale s	Saccharomycetales_fam_Incertae_se dis	*Candida*

Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*
Ascomycota	Saccharomycetes	Saccharomycetales	Trichomonascaceae	*Blastobotrys*
Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	*Coniochaeta*
Ascomycota	Sordariomycetes	Hypocreales	NA	NA
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Nectria*
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Nectria*
Ascomycota	Sordariomycetes	Hypocreales	Niessliaceae	*Eucasphaeria*
Ascomycota	Sordariomycetes	Hypocreales	Niessliaceae *	*Niesslia* *99%
Ascomycota	Taphrinomycetes	Taphrinales	Protomycetaceae	*Protomyces*
Ascomycota	Taphrinomycetes	Taphrinales	Taphrinaceae	*Taphrina*
Ascomycota	Taphrinomycetes	Taphrinales	Taphrinaceae	*Taphrina*
Ascomycota	Taphrinomycetes	Taphrinales	Taphrinaceae	*Taphrina*
Basidiomycota	Agaricostilbomycetes	Agaricostilbales	Kondoaceae	*Kondoa*
Basidiomycota	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae	*Cystobasidium*
Basidiomycota	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae	*Cystobasidium*
Basidiomycota	Cystobasidiomycetes	Cystobasidiales	NA	NA
Basidiomycota	Exobasidiomycetes	Entylomatales	Entylomatales_fam_Incertae_sedis	*Tilletiopsis*
Basidiomycota	Malasseziomycetes	Malasseziales	Malasseziaceae	*Malassezia*
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae	*Leucosporidium*
Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*
Basidiomycota	NA	NA	NA	NA
Basidiomycota	NA	NA	NA	NA
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Cystofilobasidium*
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Guehomyces*
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Udeniomyces*
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Udeniomyces*
Basidiomycota	Tremellomycetes	Cystofilobasidiales	NA	NA
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*

Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Naganishia*
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Naganishia*
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Naganishia*
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Naganishia*
Basidiomycota	Tremellomycetes	Filobasidiales	Piskurozymaceae	*Piskurozyma*
Basidiomycota	Tremellomycetes	Holtermanniales	Holtermanniales_fam_Incertae_sedis	*Holtermanniella*
Basidiomycota	Tremellomycetes	Tremellales	Bulleraceae	*Bullera*
Basidiomycota	Tremellomycetes	Tremellales	Bulleraceae	*Bullera*
Basidiomycota	Tremellomycetes	Tremellales	Bulleraceae	*Fonsecazyma*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*
Basidiomycota	Tremellomycetes	Tremellales	NA	NA
Basidiomycota	Tremellomycetes	Tremellales	Phaeotremellaceae	*Gelidatrema*
Basidiomycota	Tremellomycetes	Tremellales	Phaeotremellaceae	*Gelidatrema*
Basidiomycota	Tremellomycetes	Tremellales	Sirobasidiaceae	*Fibulobasidium*
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	*Cryptococcus*
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	*Tremella*
Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	*Tremella*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Trichosporon*
Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Tranzscheliella*

Table 3.S1 continued

Species	**Total Bats**	*Antrozous pallidus*	*Corynorhinus townsendii*	*Eptesicus fuscus*	*Euderma maculatum*	*Myotis californicus*

Total sample size for each species	450	39	83	9	8	34
NA	1	0	1	0	0	0
NA	3	1	0	0	0	1
NA	32	6	7	1	2	4
pullulans	42	7	6	1	0	5
NA	4	0	2	0	1	0
NA	1	1	0	0	0	0
populi	5	2	0	0	0	0
NA	1	0	0	0	0	0
macrosporum	6	0	0	0	0	0
viticola	2	1	0	0	0	0
NA	1	1	0	0	0	0
NA	1	0	0	0	0	0
maritima	1	0	0	0	0	0
nigrum *99%	2	0	0	0	0	0
rhododendri	1	0	1	0	0	0
NA	1	0	0	0	0	0
sideris	1	0	0	0	0	0
xenobiotica	1	0	0	0	0	1
hyalina	2	0	2	0	0	0
NA	1	0	0	0	0	0
sessilis	1	0	0	1	0	0
minioluteus	1	0	0	0	0	1
NA	2	0	0	0	0	0
NA	1	0	0	0	0	1
NA	1	0	0	0	0	0
NA	1	0	0	0	0	1
NA	1	0	1	0	0	0
fluminis	2	0	1	0	0	0
globosus	3	0	0	0	0	0
NA	1	0	0	0	0	0
hansenii, sp.6 in east	56	3	1	4	0	1
NA	1	0	0	0	0	0
NA, sp.1 in east	28	0	0	0	0	0
NA; sp.3 in east	41	0	9	0	0	1
NA	2	0	0	0	0	0
NA	2	0	0	0	0	1
NA	2	0	0	1	0	0
NA	2	0	0	0	0	0
glaebosa	1	0	0	0	0	0
pimensis	1	0	0	0	0	0
sake	1	0	0	0	0	0

buckinghamii	10	0	0	1	0	0
NA	1	0	0	0	0	0
NA	1	0	0	0	0	0
pseudotrichia	2	0	0	0	0	1
ramulariae	1	0	0	0	0	1
capensis	1	0	0	0	0	0
NA	1	0	0	1	0	0
inouyei	3	0	0	0	0	0
americana	1	0	0	0	0	0
carpini	1	0	0	1	0	0
tormentillae	1	0	0	0	0	0
malvinella	1	0	0	0	0	0
NA	2	0	0	0	0	0
psychroaquaticum	1	1	0	0	0	0
NA	1	0	0	1	0	0
washingtonensis	1	0	0	0	0	1
vespertilionis	11	0	0	0	0	6
NA	1	0	0	0	0	0
mucilaginosa	2	0	0	0	0	0
NA	1	0	0	0	0	0
NA	1	0	0	0	0	0
capitatum	2	1	0	0	0	0
pullulans	1	0	0	0	0	0
kanasensis	1	0	0	0	0	0
puniceus	1	0	0	0	0	0
NA	1	0	0	0	0	0
floriforme	1	0	0	0	0	0
magnum	5	1	2	0	0	0
NA	1	1	0	0	0	0
NA	4	0	1	0	0	2
wieringae	4	1	1	0	0	0
friedmannii	4	0	1	0	1	1
NA	2	1	0	0	0	0
NA	3	1	0	0	0	1
randhawae	1	0	0	0	0	0
capsuligena	2	0	0	0	0	2
takashimae	13	8	0	0	0	1
alba	1	0	0	0	0	0
unica	1	1	0	0	0	0
NA	1	0	0	0	0	0
carnescens	2	0	0	0	0	0
NA	1	0	0	0	0	1
NA	10	4	1	2	0	0

tephrensis	2	0	0	1	0	0
victoriae	13	6	0	2	0	2
victoriae	1	0	0	1	0	0
NA	1	0	0	0	0	0
NA	1	0	0	1	0	0
spencermartinsiae	1	1	0	0	0	0
NA	1	1	0	0	0	0
frias	2	2	0	0	0	0
encephala	1	0	0	0	0	0
NA	2	0	0	0	0	0
guehoae	1	0	0	0	0	0
moniliiforme	9	0	0	0	0	0
otae	8	0	0	0	0	0
williamsii	1	0	0	0	1	0

Table 3.S1 continued

My. ciliolabrum	*My. evotis*	*My. lucifugus*	*My. septentrionalis*	*My. thysanodes*	*My. velifer*	*My. volans*	*My. yumanensis*	*Parastrellus hesperus*
36	47	13	3	32	31	43	55	17
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	1	0	0
2	2	0	0	4	2	1	0	1
3	5	0	1	6	2	2	3	1
1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	2
0	0	0	0	0	0	1	0	0
1	2	0	0	2	0	1	0	0
0	0	0	0	0	0	0	1	0
0	0	0	0	0	0	0	0	0
0	1	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
0	0	0	0	1	0	0	1	0
0	0	0	0	0	0	0	0	0
0	0	0	0	1	0	0	0	0
0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0

0	0	0	0	0	0	0	0	0
0	0	0	0	1	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0
3	0	0	0	0	0	0	0	0
0	0	0	0	0	0	1	0	0
0	0	0	0	14	29	0	4	0
1	0	0	0	0	0	0	0	0
0	1	1	0	1	24	0	1	0
8	0	0	2	0	18	0	3	0
0	0	1	0	0	0	1	0	0
0	0	0	0	1	0	0	0	0
0	0	0	0	0	0	0	1	0
0	0	0	2	0	0	0	0	0
0	1	0	0	0	0	0	0	0
0	0	0	0	0	0	1	0	0
0	0	1	0	0	0	0	0	0
0	1	0	0	1	0	0	7	0
0	1	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0
0	0	0	0	0	0	0	0	0
0	2	0	0	0	0	0	1	0
0	0	0	0	0	0	1	0	0
0	0	0	0	0	0	0	0	0
0	1	0	0	0	0	0	0	0
0	0	0	0	0	0	1	0	0
0	0	0	0	2	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	1	0	0	4	0
0	0	1	0	0	0	0	0	0
0	2	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0
1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	1	0	0
0	0	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0

0	0	0	0	0	0	1	0	0
0	0	0	0	1	0	0	0	0
0	0	0	0	2	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1
1	0	0	0	1	0	0	0	0
1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1
0	0	0	0	1	0	0	0	0
0	0	0	0	1	0	0	0	0
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	1	2	0
0	1	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	1	0	0
0	0	0	0	0	1	1	0	0
0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
0	2	0	0	0	0	0	1	0
0	0	0	0	0	0	0	0	0
0	0	0	0	1	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	1	0	0	0	0	0	0	0
0	0	0	0	0	0	2	0	0
0	0	0	0	0	1	0	0	0
0	0	0	0	0	9	0	0	0
0	0	0	0	0	8	0	0	0
0	0	0	0	0	0	0	0	0

Table 3.S2: Results of Gaussian zero-inflated models with two response variables: Shannon Diversity Index and number of yeast colony forming units representing yeast abundance. ‘Days’ refers to how many days samples were stored before processing. ‘Cave’ represents whether samples were collected at underground sites (caves or mines). ‘Location’ refers to the state or province of sample collection in western North America. We defined ‘seasons’ as winter (December – February), spring (April – May), summer (June – August), and fall (September – October). Significant p-values (<0.05) are in bold. The zero-inflation model for the Shannon Diversity model (~1) estimate = -26.64, standard error = 44616.95, p-value= 1.

Model	Shannon Diversity Index - yeast only ~ Bat Species + Days + Location + Season + Cave			Yeast Abundance ~ Bat Species + Days + Location + Season + Cave			Yeast Abundance - zero inflation model ~ Bat Species + Days + Location + Season + Cave		
	Estimate	Standard Error	P-value	Estimate	Standard Error	P-value	Estimate	Standard Error	P-value
Intercept	0.715	0.092	**< 0.001**	2.793	0.287	**< 0.001**	-2.467	1.100	**0.025**
Corynorhinus townsendii	-0.013	0.098	0.896	0.984	0.32218	**0.002**	3.532	1.520	**0.020**
Eptesicus fuscus	0.238	0.117	**0.042**	1.203	0.36229	**< 0.001**	1.099	1.589	0.489
Euderma maculatum	0.054	0.117	0.646	0.726	0.49132	0.140	1.346	1.159	0.246
Myotis californicus	-0.128	0.071	0.071	0.038	0.2268	0.868	0.590	0.645	0.361
M. ciliolabrum	-0.118	0.092	0.2	1.236	0.296	**< 0.001**	0.805	1.045	0.441
M. evotis	-0.189	0.073	**0.009**	-0.086	0.254	0.735	0.793	0.748	0.290
M. lucifugus	-0.391	0.099	**< 0.001**	-0.519	0.316	0.101	2.454	1.266	0.053
M. thysanodes	-0.177	0.081	**0.029**	-0.858	0.341	**0.012**	1.080	0.855	0.206
M. velifer	0.075	0.142	0.594	-0.263	0.511	0.607	-1.544	118700.000	1.000
M. volans	-0.17	0.068	**0.013**	-0.578	0.284	**0.042**	0.868	0.674	0.197
M. yumanensis	-0.256	0.1	**0.011**	-0.571	0.311	0.066	2.079	1.014	**0.040**
Parastrellus hesperus	-0.2	0.08	**0.012**	-0.615	0.334	0.065	1.269	0.753	0.092
Days	-0.007	0.003	**0.015**	0.011	0.011	0.292	0.035	0.031	0.265
Cave	-0.299	0.114	**0.009**	-1.912	0.560	**< 0.001**	-0.569	1.796	0.751
Spring	-0.248	0.099	**0.012**	-0.501	0.298	0.093	-0.466	1.161	0.688
Summer	-0.421	0.071	**< 0.001**	-1.048	0.234	**< 0.001**	1.670	0.934	0.074
Winter	-0.29	0.085	**0.001**	-1.296	0.261	**< 0.001**	-20.320	4532.000	0.996
British Columbia	-0.081	0.089	0.361	0.040	0.361	0.912	0.063	0.866	0.942
California	-0.013	0.064	0.845	-0.029	0.267	0.914	0.260	0.641	0.685
Colorado	0.176	0.146	0.226	0.894	0.587	0.128	17.700	4532.000	0.997
Idaho	0.21	0.143	0.142	0.163	0.626	0.795	-25.660	183400.000	1.000
Montana	-0.068	0.149	0.647	0.599	0.622	0.336	2.299	1.869	0.219
Nevada	0.046	0.087	0.597	-0.559	0.492	0.255	1.400	1.042	0.179
Oregon	-0.045	0.065	0.488	-0.335	0.252	0.183	0.491	0.597	0.411
Texas	0.606	0.147	**< 0.001**	3.291	0.649	**< 0.001**	-1.976	2.092	0.345
Utah	-0.055	0.128	0.669	1.449	0.554	**0.009**	21.040	4532.000	0.996
Washington	-0.088	0.101	0.385	-0.686	0.406	0.091	-0.392	1.196	0.743
Wyoming	-0.04	0.157	0.798	0.384	0.630	0.542	19.850	4532.000	0.997

Chapter 4 Supplemental Data

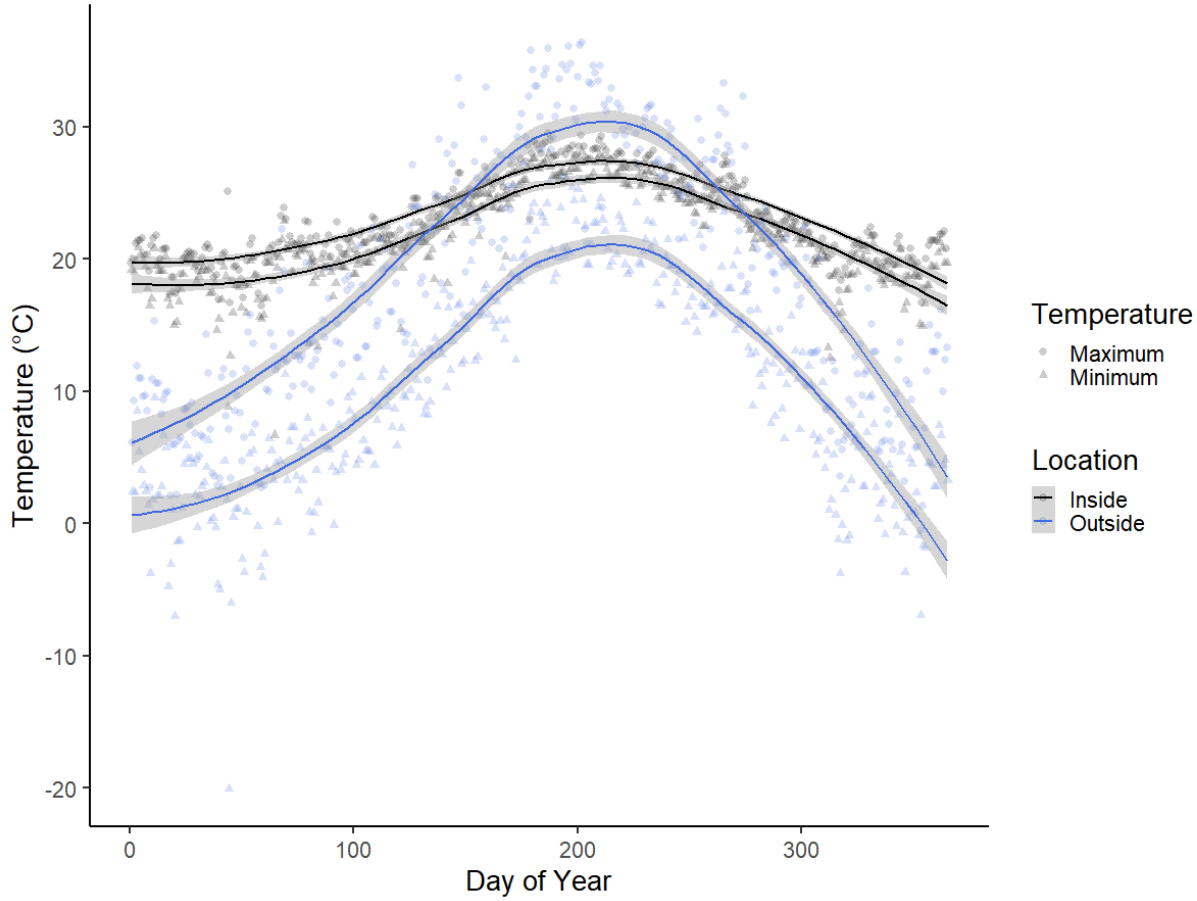

Figure 4.S4: Daily maximum and minimum temperatures recorded April 2019 – March 2020 inside and outside the captive *Eptesicus fuscus* colony in Hamilton, Ontario. Markers represent individual data points while the lines indicate the mean with 95% confidence intervals in gray shading.

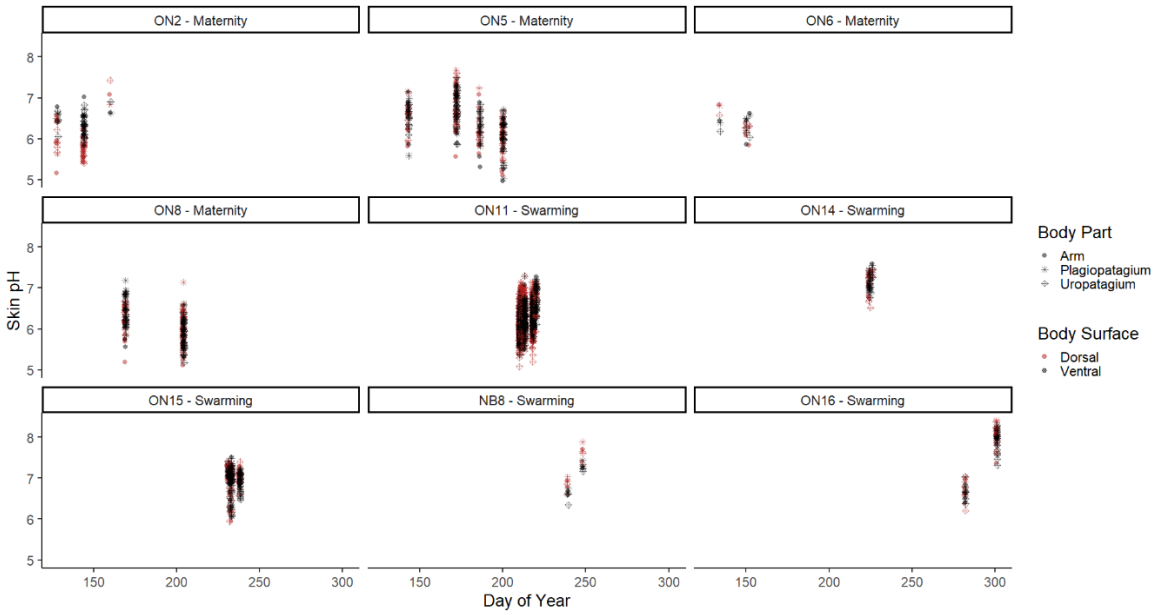

Figure 4.S5: Sites where wild bats were sampled over multiple time points. Sites that were sampled only twice and with two days or less between visits were excluded.

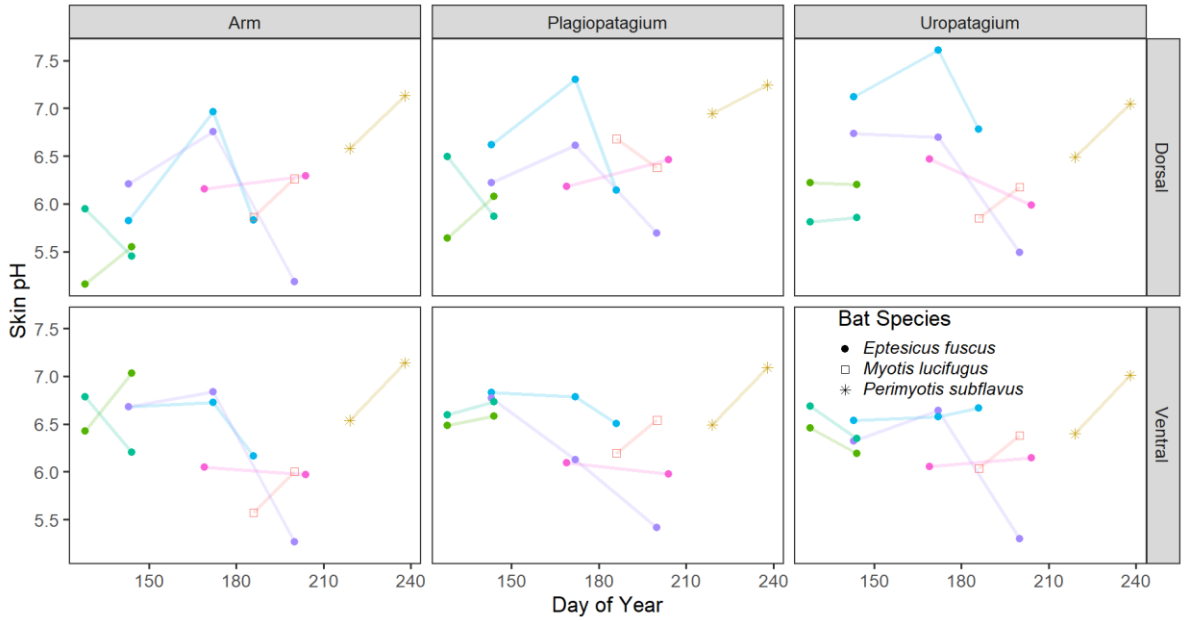

Figure 4.S6: Skin pH of seven wild bats in Ontario that were captured two to three times over summer 2019. Each color indicates an individual bat.

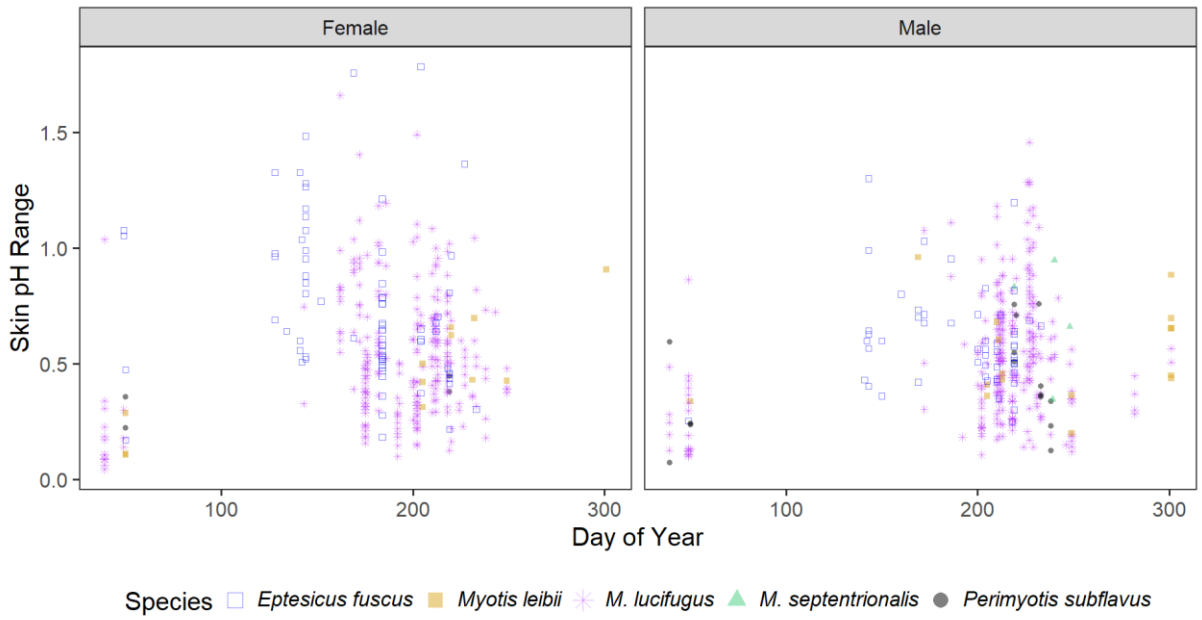

Figure 4.S7: Range in skin pH values among the six body parts measured on individual wild bats. Range was calculated by subtracting the lowest from the highest value on each bat.

Table 4.S1: Summary of previous research on the skin pH of non-human vertebrates. 'Months' indicates when measurements were taken. Whether the hair/fur of animals were shaved or clipped prior to measurement is indicated, although some animals and neonates are naturally hairless. When available, pH ranges are given in brackets in the 'mean pH' column. Articles on the skin pH of laboratory mice are representative of a larger literature body.

Reference	Location	Country	Common Name	Species
Bartels et al 1991	Zoo	Germany	36 bird species	
	Laboratory	United States	spotted salamanders	*Ambystoma maculatum*
Barnhart et al. 2020	Laboratory	United States	fire salamanders	*Salamandra salamandra*
	Laboratory	United States	eastern red-spotted newts	*Notophthalmus viridescens*
Woodhams et al 2012	Laboratory	United States	mountain yellow-legged frog	*Rana muscosa*
Litwiller et al 2006	Laboratory	Canada	mangrove killifish	*Rivulus marmoratus*
Tsui et al 2002	Laboratory	Singapore	weather loach	*Misgurnus anguillicaudatus*
Menon et al. 2019	Zoo	United States	naked mole rat	*Heterocephalus glaber*
Bourdeau et al. 2004	Domestic	France	cat, European short-haired	*Felis catus**
Szczepanik et al 2011	Domestic	unknown	cat, European short-haired	*Felis catus**

Santoro et al 2021	Domestic	unknown	cat, short-hair & Siamese	*Felis catus**
Ajito et al. 2001	Domestic	Japan	cat, Siamese	*Felis catus**
	Domestic	Germany	cat, 4 breeds	*Felis catus**
Meyer and Neurand 1991	Domestic	Germany	rabbit, 4 breeds	*Oryctolagus cuniculus domesticus**
	Domestic	Germany	guinea pigs	*Cavia porcellus**
	Domestic	United States	guinea pigs	*Cavia porcellus**
	Laboratory	United States	monkey	species unknown
Draize 1942	Domestic	United States	cat	*Felis catus**
	Laboratory	United States	rat	*Rattus norvegicus domestica**
	Domestic	United States	rabbit	*Oryctolagus cuniculus domesticus**
	Domestic	United States	dog, breed unknown	*Canis familiaris**
Bradley et al. 2016	Domestic	United States	dog, 21 breeds	*Canis familiaris**
Dunstan et al 2002	Domestic	United States	dog, 3 breeds	*Canis familiaris**
Hobi et al. 2017	Domestic	unknown	dog, 14 breeds	*Canis familiaris**
Oh and Oh 2009	Domestic	Korea	dog, beagle	*Canis familiaris**
Ruedisueli et al. 1998	Domestic	unknown	dog, 4 breeds	*Canis familiaris**
Matousek et al. 2003	Domestic	unknown	dog, mixed breed	*Canis familiaris**
Breathnach et al. 2011	Domestic	Ireland	dog, multiple breeds	*Canis familiaris**
Klinger et al. 2018	Domestic	Germany	dog, 10 breeds	*Canis familiaris**
Popiel and Nicpon 2004	Domestic	unknown	dog, 12 breeds	*Canis familiaris**
Cobiella et al. 2019	Domestic	unknown	dog, 11 breeds	*Canis familiaris**
Meyer and Neurand 1991	Domestic	Germany	dog, 5 breeds	*Canis familiaris**
Ajito et al. 2001	Domestic	Japan	dog, beagle	*Canis familiaris**
Zajac et al. 2015	Domestic	Poland	dog, 13 breeds	*Canis familiaris**
Young et al. 2002	Domestic	unknown	dog, 4 breeds	*Canis familiaris**
Roy 1954	Domestic	United States	dog	*Canis familiaris**
Santoro et al 2021	Domestic	United States	dog, 6 breeds	*Canis familiaris**
Joly 2018	Domestic	Brazil	dog, Shih Tzu	*Canis familiaris**

Urnau 2018	Domestic	Brazil	dog, golden retriever & shih tzu	*Canis familiaris**
Grono 1970	Domestic	unknown	dog, breeds unknown	*Canis familiaris**
Ferreira 2010	Domestic	Portugal	dog, 16 breeds	*Canis familiaris**
Bogacz 1992	Domestic	Poland	sheep	*Ovis aries**
Meyer and Neurand 1991	Domestic	Germany	sheep, 3 breeds	*Ovis aries**
Meyer et al. 2001	Domestic	Turkey	sheep, Merino	*Ovis aries**
	Domestic	Turkey	goat, Angora	*Capra aegagrus hircus**
Meyer and Neurand 1991	Domestic	Germany	goat, 3 breeds	*Capra aegagrus hircus**
	Domestic	Germany	pig, 3 breeds	*Sus scrofa domesticus**
Ajito et al. 2001	Domestic	Japan	pig	*Sus scrofa domesticus**
Meyer et al. 1991	Domestic	Germany	camel, dromedary	*Camelus dromedarius**
Szczepanik et al 2013	Domestic	Poland	ponies, Polish	*Equus ferus caballus**
Szczepanik et al 2012	Domestic	Poland	ponies, Felin	*Equus ferus caballus**
Meyer and Neurand 1991	Domestic	Germany	horse, 3 breeds	*Equus ferus caballus**
Koziol et al. 2017	Domestic	United States	bull	*Bos taurus**
Meyer and Neurand 1991	Domestic	Germany	cattle, 3 breeds	*Bos taurus**
Jenkinson and Mabon 1973	Domestic	unknown	Ayrshire cattle	*Bos taurus**
Fox et al. 2003	Domestic	United States	Holstein cow	*Bos taurus**
Zecconi et al 2005	Domestic	Italy	Italian Holstein cow	*Bos taurus**
Ajito et al. 2001	Domestic	Japan	cow	*Bos taurus**
Hamann et al 2002	Domestic	Germany, New Zealand	cow	*Bos taurus**
Meyer and Neurand 1991	Laboratory	Germany	laboratory rats	*Rattus norvegicus domestica**
Golynski et al 2014	Laboratory	Poland	rat, Wistar	*Rattus norvegicus domestica**
Behne et al 2003	Laboratory	United States	laboratory rats	*Rattus norvegicus domestica**
Fluhr et al. 2004	Laboratory	United States	laboratory rats	*Rattus norvegicus domestica**
Fluhr et al. 2004 part2	Laboratory	United States	laboratory rats	*Rattus norvegicus domestica**

Fluhr et al. 2004 part2	Laboratory	United States	mice, asebia-J & wild-type	*Mus musculus**
Choi et al. 2007	Laboratory	United States	hairless mice	*Mus musculus**
Hatano et al. 2009	Laboratory	United States	hairless mice	*Mus musculus**
Lee et al. 2014	Laboratory	Korea	hairless mice	*Mus musculus**
Lee et al. 2016	Laboratory	Korea	hairless mice	*Mus musculus**
Proksch et al. 2019	Laboratory	Germany	hairless mice	*Mus musculus**
Pan et al. 2010	Laboratory	Taiwan	hairless mice	*Mus musculus**
Mauro et al. 1998	Laboratory	United States	hairless mice	*Mus musculus**
Hachem et al. 2010	Laboratory	unknown	hairless mice	*Mus musculus**
Fluhr et al 2001	Laboratory	United States	hairless mice, Skh1/Hr	*Mus musculus**
Hachem et al. 2003	Laboratory	unknown	hairless mice, Skh1/Hr	*Mus musculus**
Danciu et al. 2014	Laboratory	Romania	hairless mice, SKH1	*Mus musculus**
Behne et al 2002	Laboratory	unknown	hairless mice, SKH1 & wild-type	*Mus musculus**
Hachem et al. 2005	Laboratory	United States	hairless mice, SKH1/Hr	*Mus musculus**
Tang et al. 2021	Laboratory	China	mice, BALB/c	*Mus musculus**
Jang et al. 2016	Laboratory	Japan	mice, NC/Tnd	*Mus musculus**
Sakai et al. 2014	Laboratory	Japan	mice, flaky-tail & wild type	*Mus musculus**
Moniaga et al. 2013	Laboratory	Japan	mice, flaky-tail & B6	*Mus musculus**
Wen et al. 2021	Laboratory	China	mice, C57BL/6J	*Mus musculus**

Table 4.S1 continued

Sex	Age	n	Months	Body Part	Hair/fur shaved or clipped?
unknown	unknown	152	unknown	unfeathered skin areas dorsal & ventral	Not Applicable
unknown	Juvenile	10	unknown	Dorsal	Not Applicable
unknown	Juvenile	10	unknown	Dorsal	Not Applicable
unknown	Adult	10	unknown	Dorsal	Not Applicable
unknown	Adult	24	July, August	ventral & dorsal body	Not Applicable
hermaphrodite	>1 year	14?	unknown	anterior operculum, base	Not Applicable

both sexes	Adult	16	unknown	of pectoral & caudal fins just above pectoral fins	Not Applicable
unknown	Adult	10	unknown	unknown	Not Applicable
12 male, 12 female	14 months	24	followed over 1 year	unknown	unknown
8 male, 12 female	6 months to 6 years	20	March to November	lumbar region, left axillary fossa, right inguinal region, ventral abdomen, left lateral thorax	Yes
4 male, 13 female	mean 5.7 ± 3.5	17	unknown	inguinal, axillary, and aural surfaces	unknown
unknown	unknown	10	unknown	unknown	No
14 male, 14 female	Adult	28	unknown	dorsal, lateral, abdominal, frontal, nasolabial, foot	No
sexes roughly equal	Adult	25	unknown	dorsal, lateral, abdominal, frontal, nasolabial, foot	No
sexes roughly equal	Adult	29	unknown	dorsal, lateral, abdominal, frontal, nasolabial, foot	No
unknown	unknown	98	unknown	upper & lower abdomen, axilla, inguinal, back, neck	Yes
1 male, 5 females	unknown	6	unknown	upper & lower abdomen, axilla, inguinal, back, neck	Yes
20 females	unknown	27	unknown	upper & lower abdomen, axilla, inguinal, back, neck	Yes
unknown	6 weeks to 2 years	60	unknown	upper & lower abdomen, axilla, inguinal, back, neck	Yes
12 male, 12 female	6 months to 1 year	24	unknown	upper & lower abdomen, axilla,	Yes

				inguinal, back, neck	
15 males, 19 females	few days to 4 years	34	unknown	upper & lower abdomen, axilla, inguinal, back, neck	Yes
11 male, 17 female	8 - 144 months	28	unknown	axilla, groin, ventral pinna	No
36 male	63 weeks at start of study	36	followed over 4.5 months (months not given)	unknown	unknown
7 male, 19 female	mean age 6 years	26	unknown	pinna, scapula, caudal back, abdomen	Yes
male	2-4 years	5	unknown	head, upper eyelid, nose, lower muzzle, upper muzzle, ear, upper back, lower back, tail, axilla, cranial elbow, inguinal region, interdigital skin and footpad	Yes
14 males, 22 females	1-9 years	36	unknown	flanks, dorsum of head, inner surface of pinna, inguinal & axillary regions	Yes
5 male, 3 female	Adult	8	unknown	dorsal thorax & lumbar regions	Yes
31 male, 19 female	Adult	50	unknown	right front & left hind feet	No
unknown	2-11 years	23	unknown	ventrum, tail base, inner pinna, between shoulder blades	Yes
both sexes	Adult	40	unknown	back, lumbosacral region	unknown
18 male, 12 females	mean 5.6 & 7.3 years	30	unknown	inguinal, axilla, pinna, interdigital	Yes
20 male, 20 female	Adult	40	unknown	dorsal, lateral, abdominal,	No

unknown	unknown	8	unknown	frontal, nasolabial, foot unknown	Yes
		33		lumbar region, right axillary fossa, right inguinal region, ventral abdomen, left lateral thorax, internal surface auricle, interdigits right forelimb, cheek,	
21 male, 12 female	2.5-7 years		over 24 months	nose, lateral antebrachum	unknown
16 male, 16 female	2-14 years	32	unknown	lumbar	Yes
unknown	1-14 years	243	unknown	back	unknown
5 male, 5 female	mean 5.3 ± 3.6	10	unknown	inguinal, axillary, pedal, and aural surfaces	unknown
		24		perilabial, internal and external face of the auricular pavilions, dorsal interdigital regions and ventral, armpits, neck, abdomen,	
10 male, 14 female	1-10 years		unknown	inguinal and flank regions	unknown
both sexes	under 18 months to over 7 years	54	unknown	periocular, head, perilabial, perianal, armpit, groin, abdomen, inguinal, flank, palm, plantar	unknown
52 male, 60 female	unknown	112	unknown	ear epithelial surface	unknown
		197		muzzle, pavilion auricular, dorsal & ventral interdigital,	
83 male, 98 female	1 month - 16 years		unknown	axilla, inguinal, flank	No

unknown	unknown	unknown	unknown	inside ear, inside thigh	No
most female	Adult	31	unknown	dorsal, lateral, abdominal, frontal, nasolabial	No
unknown	3-4 years	6	unknown	shoulder, flank, abdomen	No
unknown	3-9 years	4	unknown	shoulder, flank, abdomen	No
most female	Adult	16	unknown	dorsal, lateral, abdominal, frontal, nasolabial	No
14 male, 14 female	Adult	28	unknown	dorsal, lateral, abdominal, frontal, nasolabial, foot	No
unknown	unknown	12	unknown	unknown	No
unknown	unknown	4	unknown	flank, belly, back	No
6 males, 6 females	2-14 years	12	unknown	neck, shoulder, thorax, lumbar, inguinal, lip, pinna	Yes
11 male, 5 female	2-23 years	16	unknown	neck, shoulder, thorax, lumbar, inguinal, lip, auricle	Yes
12 male, 12 female	Adult	24	unknown	dorsal, lateral, abdominal, frontal, nasolabial, foot	No
male	15-84 months	55	unknown	prepuce	Not Applicable
4 male, 28 female	Adult	32	unknown	dorsal, lateral, abdominal, frontal, nasolabial, foot	No
21 castrated male, 101 female	2 months - 14 years	122	unknown	back, muzzle, udder, teat	Yes
female	Adult	99	weekly measurements January - March	teat	Not Applicable
female	Adult	50	weekly September-November	teat	Not Applicable

most female	calves & adults	23	unknown	neck, last rib	Yes
female	Adult	53	unknown	teat	Not Applicable
21 male, 21 female	Adult	42	unknown	dorsal, lateral, abdominal, frontal, nasolabial, foot	No
12 male, 12 female	unknown	24	unknown	left side of chest	Yes
unknown	0-7 days	11 to 15	unknown	flank	Not Applicable
both sexes	0-6 days	9	unknown	back and flank	Not Applicable
unknown	0-6 days	13	unknown	unknown	Yes
unknown	Adult	12	unknown	unknown	Yes
male	8-12 weeks & 12-15 months	9?	unknown	flank	Not Applicable
female	6-8 weeks	unknown	unknown	unknown	Not Applicable
female	6 weeks	36	unknown	not measured	Not Applicable
female	unknown	30	unknown	dorsal	Not Applicable
male	6-8 weeks	unknown	unknown	flank	Not Applicable
female	8 weeks	6	unknown	dorsal	Not Applicable
male	>3 months	8?	unknown	flank	Not Applicable
unknown	6-8 weeks	50?	unknown	flank	Not Applicable
male	8-12 weeks	23?	unknown	flank	Not Applicable
male	6-8 weeks	30?	unknown	flank	Not Applicable
male	10-12 weeks	15	unknown	unknown	Not Applicable
male	8-12 weeks	28	unknown	flank	Not Applicable
male	6-8 weeks	15?	unknown	flank	Not Applicable
male	7-9 weeks	25	unknown	dorsal	Yes
male	5-12 weeks	16?	unknown	dorsal	No
female	5-46 weeks	unknown	unknown	flank	unknown
female	1-7 weeks	8	unknown	unknown	unknown
female	6-8 weeks	46	unknown	both flanks	unknown

Table 4.S1 continued

Mean ± Standard Deviation	**Conclusions**
5.6 ± 0.3 dorsal, 5.6 ± 0.3 lateral, 5.6 ± 0.3 ventral, (4.93 to 6.03)	no difference among body regions or species

5.72 ± 0.38 5.73 ± 0.30 5.57 ± 0.54	no difference among species so skin pH does not explain differences in susceptibility to *Batrachochytrium salamandrivorans*
7.07 ± 0.3 ventral, 6.91 ± 0.4 dorsal	No correlation of skin pH with *Batrachochytrium dendrobatidis* infection load on dorsal but weakly positively correlated with ventral
7.28 ± 0.08 immersed, 7.84 ± 0.05 air exposed 1 hour, 7.49 ± 0.05 air exposed 11 days	Skin pH lower than the surrounding water (8.1pH). Skin pH did not differ among body parts but did differ between immersed & air exposed fish which may be due to ammonia volatilization.
6.65 ± 0.09 control, 6.61 ± 0.12 ammonia exposed, 8.23 ± 0.18 air exposed	Submerged fish had higher skin pH vs. air exposed fish (water 7.2-8.07pH) which may be due to ammonia volatilization. Skin pH did not differ among body parts.
6.49 ± 0.14 at 23% humidity, 6.41 ± 0.52 at 65% humidity	skin pH did not differ between animals in high versus low humidity environments
(6.6 - 7.4)	skin pH did not differ by sex or diet; skin pH did differ by sexual status
6.94 male, 6.54 female	skin pH significantly differed among body parts. Males had more alkaline skin than females
not reported	skin pH did not change with intradermal treatment of heat-killed actinomycetales for feline atopic syndrome
7.07 ± 0.13	
5.92 ± 0.25 (5.64-6.4) dorsal	No difference between the sexes.
5.42 ± 0.34 (4.97-6.25) dorsal	No difference between the sexes.
5.32 ± 0.16 (5.06-5.55) dorsal	No difference between the sexes.
5.5 (4.26 - 7.17) 6.42 (5.5 - 7.52) 6.43 (5.57 - 7.44) 6.48 (5.74 - 7.51) 6.71 (5.97 - 7.5) 7.52 (5.18 -9.18)	Nape or back of the neck is most acidic. Younger individuals of a given species were slightly more acidic than adults.
medians of axilla 7.1, groin 6.8, pinna 6.8	Severity of atopic dermatitis skin lesions negatively correlated with pH. Shannon Diversity Index of skin bacteria had a weak positive correlation with pH.
6.7-8.9	Skin pH varied among dog breeds and decreased over the length of the study
7 ear, 7.5 inguinal, 7.8 back, 7.6 shoulder	all dogs had atopic dermatitis. Applying a topical product containing free fatty acids, cholesterol, and ceramides twice weekly did not change pH
7.7 ± 0.7 (6.7-8.7)	skin pH significantly differed among body parts
7.48 ± 0.04 flank, 8.1 ± 0.06 head, 6.11 ± 0.03 pinna	skin pH significantly differed among body parts & breeds. Males had more alkaline skin than females
7.55 (6.3-9)	skin pH did not differ among body parts. Spraying skin with acidic liquid decreased skin pH to <5 for 29-35 hours. All animals were neutered.

6.52 ± 0.34 diseased, 6.27 ± 0.4 healthy	skin pH was higher in dogs with lymphocytic–plasmacytic pododermatitis but was not associated with isolation of *Staphylococcus pseudintermedius*. There was a significant association of skin pH with age & breed
7.3 ± 0.9 (4.9-9.5)	all dogs had atopic dermatitis
7.4 ± 0.34 (6.91-8.02) healthy, 8.11 ± 0.48 (7.39-9.14) diseased	dogs with pyoderma had higher skin pH versus healthy dogs. Skin pH decreased after application of acidic topical products and improved skin quality. Increased skin pH was associated with occurrence of *Staphylococcus* spp.
pinna: 6.75 ± 0.76 healthy, 7.22 ± 0.9 diseased (5.47-9.11 across all sites)	dogs with atopic dermatitis had higher skin pH than healthy dogs. Skin pH was positively correlated with severity of skin lesions.
Dorsal: 5.9 ± 0.45 (5.5-6.6) unexcited, 7.89 ± 1.07 (6.6-9.39) excited	No difference between the sexes. Excited dogs had higher skin pH versus unexcited.
7.75 ± 0.71 (5.18-9.18)	
7.6 lumbar	severity of atopic dermatitis skin lesions was positively correlated with skin pH in some body parts
7.5 ± 0.07 beagle, 7.68 ± 0.1 fox terrier, 7.37 ± 0.18 labrador retriever, 8.07 ± 0.16 manchester terrier	Skin pH differed among breeds
8.2-9 dogs with eczema, 5.5-7.2 healthy dogs	most cases of eczema occur from May-October. Apocrine sweat is alkaline.
6.6 ± 0.3 inguinal, 7 ± 0.3 axilla, 6.7 ± 0.3 pinna, 6.9 ± 0.3 interdigital	skin pH did not change with treatment of lactobacilli spray
7.1 ± 0.77 flank, 6.36 ± 0.49 ear in females; 6.7 ± 0.78 flank, 6.87 ± 0.84 ear in males (5.38-8.84)	no difference in skin pH male vs. female or castrated vs. intact. Skin pH varied among body parts
6.5 ± 0.5 (6.07-6.73) golden retriever, 6.3 ± 0.8 (5.71-6.94) Shih Tzu	no difference between sexes or breeds but older dogs had more alkaline skin vs. younger dogs and pH varied among body parts
6.1 male, 6.2 female (4.6-7.2)	dogs with otitis externa had different skin pH, some higher and some lower depending on whether the disease was acute, chronic, proliferative, or parasitic
6.6 (4.9-8.3)	skin pH did not differ between age groups or sexes but did differ among body parts & breeds
7.4 in dirty conditions, 6.7 in good conditions, 7.2 (5.9-8.79) ear, 7.0 (5.6-8.8) thigh	
Dorsal: 9.11 ± 0.44 (8.64-9.86) long fleece, 6.81 ± 0.34 (6.34-7.1) short fleece	No difference between the sexes.
8.98 ± 0.56 (7.84-9.86) back, 9.32 ± 0.64 (8.06-10) flank, 8.99 ± 0.85 (6.9-9.78) abdomen	
5.93 ± 0.28 (5.68-6.32) back, 6.01 ± 0.38 (5.62-6.61) flank, 6.18 ± 0.56 (5.35-7.0) abdomen	measurements were of the fleece at different distances from the skin surface. High pH attributed to accumulation of wool grease.

5.57 ± 0.29 (4.85-6.06) dorsal	No difference between the sexes.
6.28 ± 0.39 (5.65-6.86) dorsal	No difference between the sexes.
7.22 ± 0.28	
8.14 ± 0.82 back, 8.69 ± 0.1 flank, 8.31 ± 1.17 belly	
8.01 male, 7.39 female	skin pH significantly differed among body parts but not sexes
7.26 male, 7.4 female	skin pH differed among body parts but not between sexes.
6.07 ± 0.24 (5.45-6.54) dorsal	No difference between the sexes.
8.45 (6.35-9.46)	preputial pH of silage-fed bulls was significantly lower than that of bulls fed forage only or grain-supplemented diets due to diet's affect on urine acidity
Dorsal: 6.68 ± 0.27 (6.2-7.21) female, 8.27 ± 0.31 (8-8.8) male	Skin pH higher in males versus females.
	Skin pH higher in males versus females. Skin pH significantly differed among body parts but not with age.
6.1 ± 0.04 (5-7.6) young (<24 months), 5.62 ± 0.05 (4.5-7.6) adult	Ventral surface more acidic vs. dorsal. No relationship between skin pH & skin temperature.
7.18 ± 0.64 treatment, 7.53 ± 0.46 control	teat skin pH lowered after treatment with acidic disinfectants & also after milking. Teat pH did not differ among months.
means from 5.6-6.2	teat pH differed when treated with different teat-dip products
8.46 ± 0.57 calves, 7.58 ± 0.61 heifers, 6.86 ± 0.23 milking cows	skin pH differed among body parts. Skin with hair had higher pH versus skin without hair. Juveniles have higher skin pH than adults.
6.7 ± 0.4 New Zealand; 7.2 ± 0.4 Germany	no influence of milking interval on skin pH. Treating teats with acidic sanitiser lowered skin pH.
6.9 ± 0.69 (6.05-8.06) dorsal	No difference between the sexes.
5.48 ± 0.28 male, 5.97 ± 0.5 female	male skin pH changed more than female pH after treatment with methimazole
6.9 one day, 5.7 seven days	neonate rat skin is alkaline but reaches adult levels one week after birth. Acidification of neonate skin starts at the stratum granulosum/stratum corneum interface then proceeds outward to the skin surface.
5.8-6.6	The delayed acidification of neonatal skin results in abnormalities in permeability barrier homeostasis and skin integrity, likely due to pH induced modulations in enzyme activity.
6.63 ± 0.02 on days 0-1, 5.35 ± 0.02 on days 7-8 after birth, 5.9 ± 0.08 adults	both secretory phospholipase A2 (sPLA2) and NHE1 activity contribute to, but do not completely account for, postnatal acidification of skin. Neonates have more alkaline skin than adults but reach adult levels after 5-6 days.
6.2 ± 0.29 asebia-J, 6.44 ± 0.13 wild-type	sebaceous gland products & histidase activity are not required for skin acidification

5.3 ± 0.4 young, 5.9 ± 0.1 old	older mice have higher skin pH than younger mice. pH-dependent changes in enzyme activity account for diminished skin barrier function in old mice. Normal function is restored by acidifying skin.
5.7	maintenance of an acidic SC pH largely prevents the emergence of the macroscopic and functional abnormalities in oxazalone-induced atopic-like dermatitis
not measured	application of acidic cream (2.8pH) prevented development of atopic dermatitis lesions compared to application with neutral cream (7.4pH)
not measured	mice with atopic dermatitis treated with acidic cream (3.5-5.5pH) had fewer skin lesions, lower eczema scores, decreased transepidermal water loss, and increased skin hydration compared to mice treated with neutral cream (7.4pH)
5.8	skin pH increases after tape-stripping (skin barrier distruption). Application of 7pH buffer on disrupted skin resulted in increased skin pH, increased transepidermal water loss, & inflammation while application with pH4 buffer attenuated these changes.
not reported	skin pH increased by 1.5-8 units after laser treatment
not reported	barrier recovery proceeds normally at an acidic pH (5.5), while recovery is delayed at neutral pH
5.5-6	Hyperacidification (to <5pH) of acutely disrupted skin accelerates barrier recovery attributable to enhanced lipid processing
5.86 ± 0.21, 6.41 ± 0.2 application of neutral buffer, 5.72 ± 0.06 with acidic buffer	skin pH increased with topical application of an inhibitor of secretory phospholipase activity or with application of a neutral buffer, both of which resulted in decreased barrier function. Application of an acidic buffer normalizes barrier function.
5.5 baseline, 7.7-8pH after superbase application	applying topical superbases acutely increased skin pH and skin integrity and cohesion decreased because serine protease activity increased with skin alkalinization and decreased with skin acidification. Acute increase in skin pH also reduces the activity of certain key lipid-processing enzymes.
6.1-6.4	exposure to UVB radiation increased skin pH over 10 weeks by ~0.5 units but this was not significantly different from unexposed mice.
5.75 NHE1 +/+, 6.01 NHE1 -/-	NHE1 knowout mice had higher skin pH vs. wild-type & the difference was more pronouced in deeper skin layers vs. the surface. Barrier recovery is inhibited in knockout mice or when NHE1 is inhibited pharmacologically, but is restored with application of topical acids.
5.5 increasing to 7.3 with treatment	mice treated with a topical superbase (12.8pH) had increased skin pH over 2 weeks compared to mice

6.6	treated with a neutral preparation, and this provoked abnormalities in skin due to sustained serine protease activity & decreased lipid-processing enzyme activity repeated application of 2, 4-dinitrofluorobenzene increased skin pH while applications of conjugated linoleic acid reduced it which accelerated barrier recovery.
6pH at 5 weeks	skin pH increased as eczema worsened; pH decreased with age. Treatment with topical acid lowered skin pH to 4.5-5.5pH & improved eczema symptoms & skin barrier function. Treatment with topical alkaline products increased skin pH to 8.5 & worsened eczema symptoms.
6 wild type, 5.7 flaky tail	Defective maintenance of low skin pH is correlated with emergence & exacerbation of atopic dermatitis
5.3 increasing to 5.8 with dermatitis & 6.8 with mite-induced dermatitis	skin pH & protease activity increased with the development of dermatitis but the increase was attenuated by the application of a protease-activated receptor-2 antagonist. Filaggrin deficiency has a minor effect on skin pH. Skin inflammation increases skin pH.
not given	skin pH did not change with topical application of Hirudoid cream or glucocorticoids

* Studies did not report scientific names

Table 4.S2: Sample sizes for each bat species in each month. The province where bats were measured is indicated after the sample size O=Ontario, N= New Brunswick, P=Prince Edward Island, Q=Quebec. Captive *Eptesicus fuscus* were sampled in Hamilton, Ontario. F = female and M = male.

Species	**Captive *E. fuscus***		**Wild *E. fuscus***		***Myotis lucifugus***		***M. leibii***		***M. septentrionalis***		***Perimyotis subflavus***	
Sex	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
January	53	24										
February	58	24	4 Q	1 Q	13 O, 3 Q	5 O, 15 Q	3 Q	1 Q			2 Q	2 O, 2 Q
March	63	23										
April	8	18										
May	16	10	23 O	10 O	2 O							
June	15	6	3 O	7 O	29 O, 35 P	3 O		1 O				
July	16	9	9 O, 27 N	19 O	76 O, 32 N	86 O, 2 N	3 O	4 O				
August	39	14	10 O, 1 N	16 O, 1 N	59 O, 6 N	74 O, 66 N	4 O	2 O		1 O, 2 N	2 O	11 O

September	63	25			4 O	10 O, 2 N	1 O	2 O		1 N		
October	60	26				6 O	1 O	6 O				
November	40	20										
December	53	20										
Total	**484**	**219**	**77**	**54**	**259**	**269**	**12**	**16**	**0**	**4**	**4**	**15**

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