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Elucidating The Sloth Hair Microbiome: A Metagenomic Comparison Of Two- And Three-Fingered Sloths

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ELUCIDATING THE SLOTH HAIR MICROBIOME: A METAGENOMIC COMPARISON OF TWO- AND THREE-FINGERED SLOTHS

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ABSTRACT

Sloths are unusual mobile ecosystems containing a high diversity of symbionts living and growing in their fur. These symbionts include poorly studied algae, arthropods, fungi, and bacteria, making sloths likely reservoirs of unexplored biodiversity. I aim to identify gaps and eliminate misconceptions in our knowledge of sloths and their symbionts, and to identify key questions to spur future research into the functions and roles of sloths within a broader ecological and evolutionary context. I also seek to position the sloth fur ecosystem as a model for addressing fundamental questions in microbial and metacommunity ecology. I used whole-community shotgun metagenomic sequencing to investigate and clarify the genetic diversity of the prokaryotic and eukaryotic microbes in the hair of two sloth species, *Bradypus variegatus* and *Choloepus hoffmanni*, during the dry season in Costa Rica. Analysis of whole community sloth hair metagenomes from the shoulder and head of 11 sloths revealed microbial communities that are far more diverse than previously recognized on sloth hair and showed differences in microbiomes based on sloth species. The abundance of cyanobacteria and green algae shotgun metagenomic sequencing revealed in sloth fur complicates the previously held belief that the green alga *Trichophilus welckeri* was responsible for the green coloration of three-fingered sloths. I demonstrate that whole-community metagenomic sequencing greatly increases the known diversity of microorganisms in the sloth hair ecosystem.

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INTRODUCTION

Sloths are slow moving "mobile ecosystems" with multi-trophic assemblages of organisms from a hierarchy of different taxa. I define an ecosystem to be a complex network of interconnected parts, such as species and abiotic components, that function as an ecological unit in a particular unit of space. The sloth and its fur can be considered a mobile ecosystem because of its complex and highly diverse community of epibionts that interact with each other and with abiotic factors, such as temperature gradients, nutrient availability, and moisture, within the space defined by the exterior of the sloth, which moves slowly through the larger forest ecosystem. They are unique systems to investigate questions in host-epibiont/host-microbiome ecology and coevolution within an unusual spatiotemporal/movement regime not typically accessible by sessile organisms or fast-moving animals.

Sloths spend much of their lives hanging from trees in Central and South America and are unique in that they have the slowest metabolisms of all mammals (Pauli et al., 2016). There are six extant species of sloths in two genera: two-fingered (Family Choloepodidae, *Choelopus spp*.) and three-fingered (Family Bradypodidae, *Bradypus spp*.) (Slater et al., 2010). Historically, the names "two-toed" and "three-toed" have been used, although this is a misnomer; I use "two-fingered" and "three-fingered" because all sloths have three toes but differ in the number of fingers they have on their upper limbs. Despite both genera being slow-moving arboreal folivores, two- and three-fingered sloths are actually very different as revealed by molecular,

morphological, and behavioral data (Figure 1, Table 1). Recent mitogenome and ancient collagen DNA phylogenetic analyses have revealed that these two sloth genera diverged between 27 and 34 million years ago (Figure 1; Delsuc et al., 2019; Presslee et al., 2019) even though they have convergently evolved similar traits, such as modified hands and feet into hook-like appendages for arboreal locomotion and suspensory posture, which were not seen in extinct fossil sloths (Nyakatura, 2012; Table 1). Both sloth genera host a curious array of largely unexplored symbioses (i.e., persistent, physical associations; Bronstein, 2015) involving taxonomically diverse microorganisms and arthropods in a multi-trophic assemblage that live within their fur or "pelage" (Aiello, 1985; Gilmore et al., 2001; Suutari et al., 2010; Higginbotham et al., 2014). The structure of sloth hair is also unusual, being characterized by cracks or grooves that are hypothesized to facilitate algal growth (Aiello, 1985; Suutari et al., 2010), which is the basis for a distinct green coloration of sloths in the wild.

The movement of sloths throughout their range and up-and-down the canopy column may connect and disperse fur symbionts between very different ecological niches. As sloths are scattered across the tree canopy, finding, catching, and studying sloths can be experimentally challenging. However, with recent advances in GPS tracking and remote-sensing/monitoring technology (Kays et al., 2015; Lennox et al. 2017; Neethirajaran, 2017; Taylor et al., 2017; Hughey et al., 2018; Shipley et al., 2018; Ripperger et al., 2019; Williams et al., 2019), it may now be easier and more feasible to pursue continuous monitoring studies of sloths that are otherwise difficult to follow by traditional search-and-catch methods. These capabilities may make sloths—along with their entourage of microbial and arthropod symbionts—a tractable model for exploring questions of epibiont transmission and context-dependency of the symbiont

community depending on seasonal changes and habitat differences across their large geographical range.

I aim to highlight how studying sloths and their epibionts may be useful in addressing fundamental questions in microbial and metacommunity ecology, microbiome science, and the evolution of symbioses. I summarize what is known about the basic biology of sloths as it relates to their symbionts, and review evidence (or lack thereof) in support of several speculative conclusions that have accrued in the literature and that have unfortunately led to misconceptions now canonized in the popular media (Meier, 2013; Graham, 2014; Greenwood, 2014; Woollaston, 2014). I aim to challenge speculations that lack clear empirical support, articulate gaps in our understanding of the sloth as a mobile ecosystem (focused particularly on sloth fur as an ecosystem), and make suggestions for future sloth research directions.

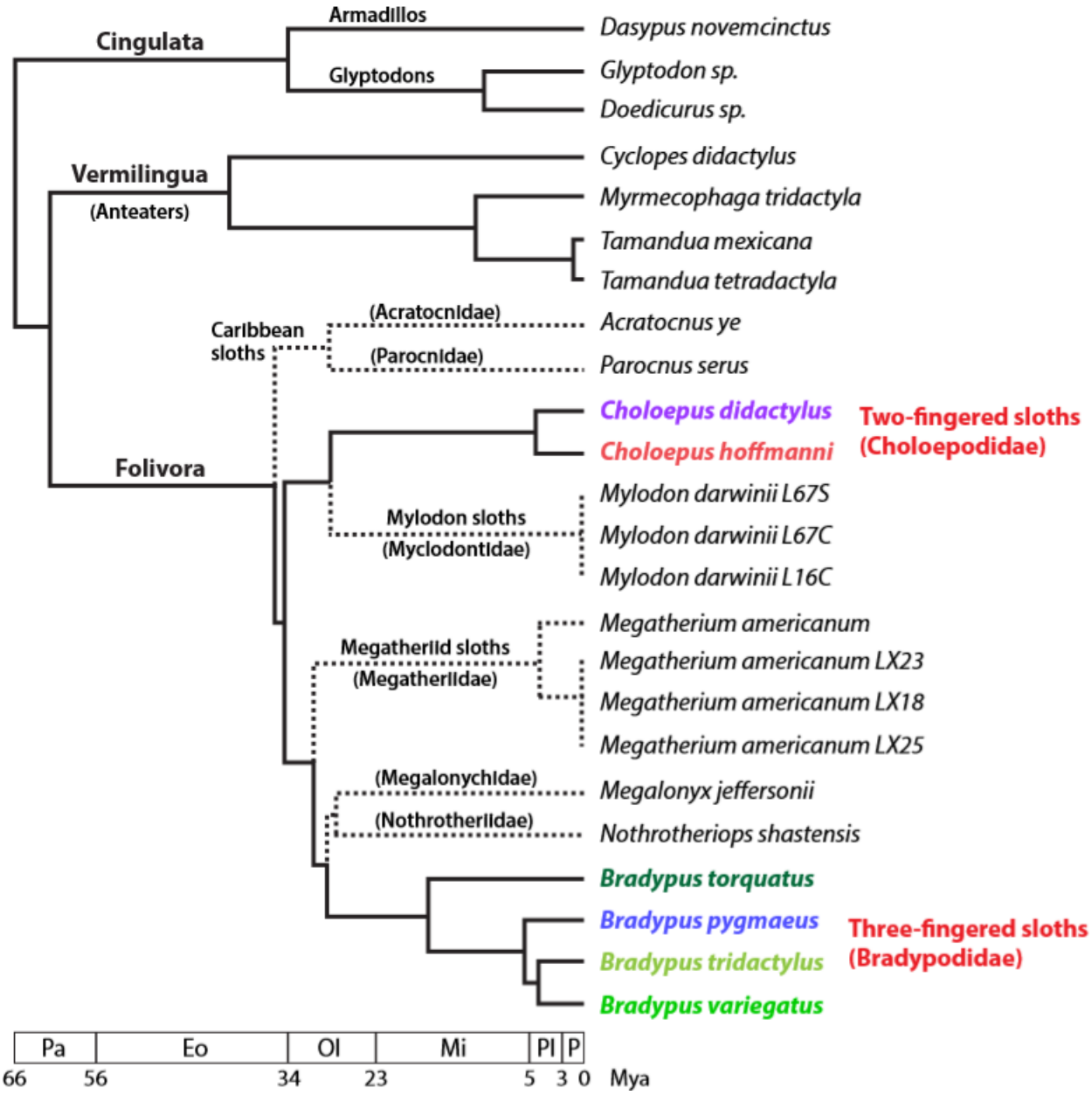


Figure 1. Phylogeny of sloths and their relatives, anteaters and armadillos, with approximate time-scalings for branches. Dashed lines indicated extinct lineages or species. Synthesized from Delsuc et al. (2019) and Presslee et al. (2019).

Table 1. Comparison of two- and three-fingered sloth characteristics. Synthesized from Aiello, (1985), Britton (1941), Falconi et al. (2015), Feldhamer et al. (2015), Goodwin (2014), Higginbotham et al. (2014), Montgomery & Sunquist (1978), Pauli & Peery (2012), Pauli et al. (2014), Pauli et al. (2016), Peery & Pauli (2012), Ramirez et al. (2011), Urbani & Bosque (2007), and Vaughan et al. (2007). It should be noted that the home range sizes of *B. variegatus* and *C. hoffmanni* were studied exclusively in a cacao agroecosystem (Ramirez et al., 2011; Vaughan et al., 2007) and thus may not be representative of the home ranges of their species. Additionally, the home range sizes of *Bradypus tridactylus*, *Bradypus pygmaeus*, and *Choloepus didactylus* have not been studied.

Two-fingered sloths	**Three-fingered sloths**
Gross Anatomy/Morphology	
2 forelimb fingers	3 forelimb fingers
5-8 neck vertebrae	8-9 neck vertebrae
Up to 8.5 kg	Up to 4.5 kg
Similar limb length	Forelimbs longer than hindlimbs
No tail	Small tail
Caniniform premolars	Only cylindrical teeth
Behavior & Range	
No basking behavior	Basking behavior
Vigorous self-defence	Minimal self-defence
Nocturnal	Cathemeral (sporadic activity over 24 hrs)
Promiscuous	Polygynous
Home range: *B. variegatus*: male mean – 21.52 ha; female mean – 1.69 ha *B. torquatus*: mean 9.8 ha *B. tridactylus*: size unknown *B. pygmaeus*: size unknown	Home range: *C. hoffmanni*: male mean – 9.18 ha; female mean – 6.45-7.1 ha *C. didactylus*: size unknown
Physiology & Diet	
Third slowest metabolism of all mammals	Slowest metabolism of all mammals
10 month gestation	5-6 month gestation
Diet mostly leaves, but also fruits, eggs, and insects	Diet almost exclusively leaves
Fur-related	
Visible algal growth in hair, 4 known genera	Visible algal growth in hair, 6 known genera
Fungal genera not clear	16 fungal genera identified
Longitudinal hair grooves	Transverse hair cracks

THE SLOTH AS A MODEL MOBILE ECOSYSTEM

All animals possess an assemblage of other species that live on or within them, the majority of which are microbial. When found within (as with gut microbiomes) these species often have a profound influence on host biology (McFall-Ngai, 2015; Barko et al., 2017). As with other mammals, the gut microbiome of sloths is believed to play an important role in sloth health and be influenced by diet (Delsuc et al., 2014; Dill-McFarland et al., 2016). However, it is the rich diversity of epibiotic symbionts on sloth fur that is most distinctive about the sloth holobiont (host + associated biota). Unlike the gut microbiome, which is shielded from the environment except through host-driven dietary intake, the sloth fur ecosystem is open to the larger forest ecosystem through which the sloth moves. This fur system is also much more than just the fur microbiome. In addition to eukaryotic microorganisms, a variety of arthropods are an integral part of the fur multi-trophic community. Similar to the pitcher plant (Boynton, 2012; Miller et al., 2017; Bittleston et al., 2018), which contains an elaborate food web of predators, prey, and detritivores that reside within a leafy "cup" and is an entire ecosystem unto itself, sloth fur is an ecosystem containing many species and trophic levels, and is relatively self-contained.

The colonization process of sloths' fur and skin is unknown but may be driven by the ecology of the skin/hair, endogenous host factors, and exogenous environmental factors as in humans (Grice & Segre, 2011). The sloth fur ecosystem likely has a layered structure, similar to the canopy structure of a species-rich grassland (Lane et al., 2000) or the stratified communities in microbial mats (Stolz, 2000) in which organisms are organized based on gradients in temperature or light penetration. Local conditions may be more stable closer to hair follicles and skin where it is warmer and dimmer, compared to those at the ends of hair tips that are more

exposed to the elements. Like trees that are colonized by microbes in their phyllosphere (foliar habitat) and by fungi and algae in lichens on tree bark, microbes of the sloth fur ecosystem may be fundamental to the well-being of the sloth and serve as the foundation for recruiting and assembling taxa from higher trophic levels. Unlike trees, however, sloths are mobile. Given the complex but compact hierarchical web of microorganisms within the sloth fur ecosystem, and the frequent interactions with hundreds of species of trees that sloths have by moving slowly through the forest canopy (Montgomery & Sunquist, 1975; Vaughan et al., 2007), sloths may be vectors of dispersal unlike any other animal and may provide a unique opportunity to bridge micro- and macro-ecological concepts (Prosser et al., 2007; Antwis et al., 2017; Shade et al., 2018).

1. Sloth Movement and Geographical Range

Sloths are essentially slow-moving ecosystems that interact with their environment, perhaps facilitating the migration of organisms to and from sloths as they move from tree-to-ground and tree-to-tree in the forest canopy. Although it is commonly thought that sloths are fairly stationary, they have been observed to move regularly throughout the forest. In one study, Hoffmann's two-fingered sloth, *Choloepus hoffmanni*, moved 38 meters or more between daily locations in 54% of radio-telemetry observations, while 11% of measures showed the brown-throated three-fingered sloth, *Bradypus variegatus*, to move >38 meters per day (Sunquist & Montgomery, 1973). The majority of these movements were found to occur during bouts of activity lasting 2-6 hours. *B. variegatus* tends to stay in trees for an extended period over days and nights whereas *C. hoffmanni* appear to spend little time at a single location during the night and move relatively longer distances (Sunquist & Montgomery, 1973). In a cacao agroecosystem, *C. hoffmanni* was found in 101 different tree species and *B. variegatus* in 71

(Vaughan et al., 2007). The home range size for sloths varies depending on sex and species (Table 1), but it is clear that they move throughout the larger ecosystem, interact with many species of trees, and may thus encounter numerous species of microorganisms and fauna in the process. Whether sloth fur microbes are transmitted vertically or horizontally via interactions with their environment (largely trees) is an open question, however it is likely that the fur microbiome is influenced by the phyllospheres they interact with. Geographically, sloths are found throughout Central and South America and the extent of species range overlap varies (Figure 2).

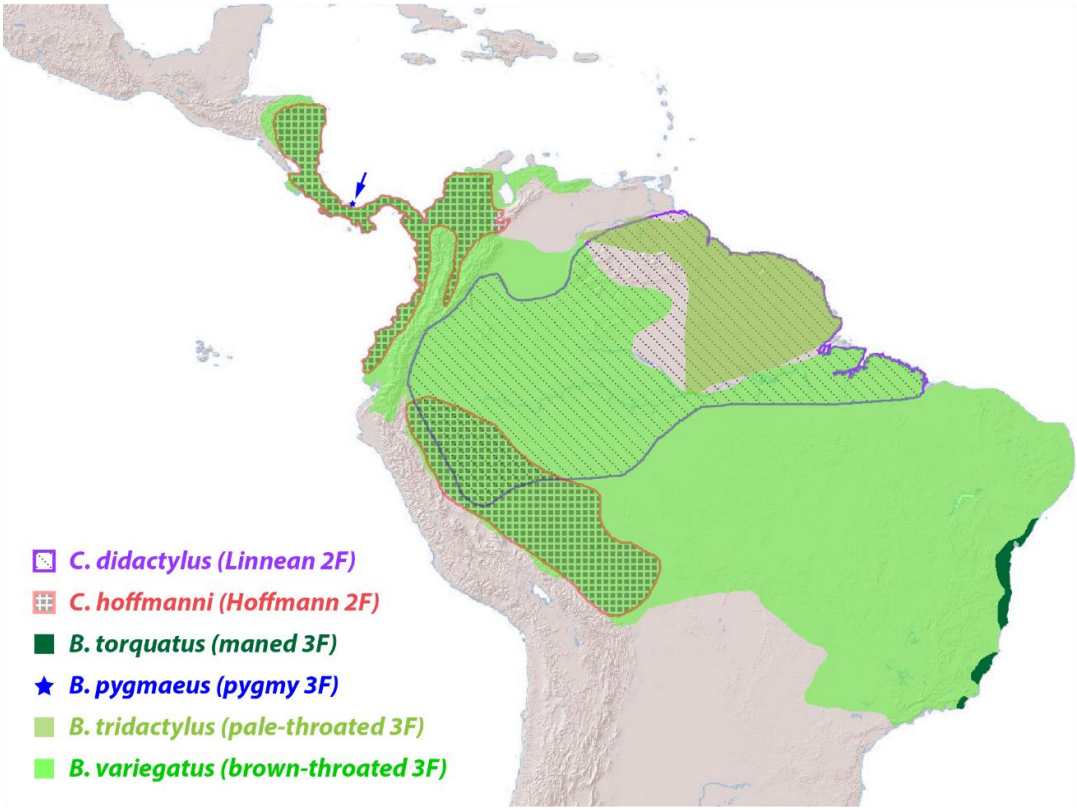


Figure 2. Distributional range of extant two-fingered (2F) and three-fingered (3F) sloth species across Central and South America. Synthesized from data of Chiarello & Plese (2014), Plese & Chiarello (2014), Chiarello & Moraes-Barros (2014a), Voirin et al. (2014), Chiarello & Moraes-Barros (2014b), and Moraes-Barros et al. (2014) available at https://www.iucnredlist.org/.

2. Convergently Evolved Behaviors and Morphologies

Two- and three-fingered sloths have many similar traits that are hypothesized to have convergently evolved (Table 1). Both groups of sloths have evolved slow metabolisms, suspensory posture, a mainly folivorous diet, long, sharp claws for gripping branches and for territorial fights, and a modified skeletal structure to suit their slow, arboreal lifestyle (Mendel, 1981; Mendel, 1985; Miller, 1935; Montgomery & Sunquist, 1978; Nyakatura, 2012; Nyakatura & Fischer, 2011; Olson et al., 2018; Pauli et al., 2016). Suspensory posture, and the many anatomical adaptations that arise for efficient suspensory locomotion in trees, are the most clearly convergent traits, given that no known fossil sloths were considered suspensory (Nyakatura, 2012). While it is not clear if ground sloths had cracked/grooved hair, this distinctive trait of all sloth species, which may facilitate algal growth, has not been found for any other mammal, including the closest relatives of sloths, armadillos and anteaters (Aiello, 1985; see Sloth Hair Structure and Algal Growth below). The only other known mammals with epibiotic algal growth are polar bears in zoos (Lewin & Robinson, 1979) and manatees (Bledsoe et al., 2006), although they do not appear to have hair with cracks/grooves. It is unclear if such crevices are examples of a coevolved adaptation or a consequence of some pre-existing trait that facilitates a symbiotic association (Anderson, 2015).

3. Transmission of Fur Symbionts

It is thought that sloth algae, the sloth fur epibiont that has been most studied, are transmitted vertically, from mother to baby (Beebe, 1926; Britton, 1941; Suutari et al., 2010), although this has not been directly tested. Moreover, horizontal transmission from the

environmental species pools to the sloth cannot be ruled out. A mixed mode of epibiont transmission is likely, given that vertical and horizontal modes represent extreme cases (Rosenberg & Zilber-Rosenberg, 2018). Obligate symbionts generally rely on vertical transmission (Rosenberg & Zilber-Rosenberg, 2018), although there is no data on whether the algae on sloths are obligately or facultatively associated. Classifying interactions between sloths and their epibionts and their degree of dependency will go hand-in-hand with understanding the mode of transmission of each epibiont. This could be done by frequently sampling sloth hair from a mother and baby sloth throughout the care of the baby, and after the juvenile has been separated from the mother. Environmental microbiota (e.g., the phyllosphere) and the sloth fur ecosystem may be mutually shaped (or mixed) by sloths traversing and interacting with the forest canopy. Sampling the bark and leaves of trees where sloths are found in tandem with sloth hair collection throughout a sloths' life would help clarify the potential for horizontal transmission between sloths and their environment.

Sloths are considered solitary (Soares & Carneiro, 2002; Taube et al., 1999; S. Trull, unpublished data) and they generally don't interact with other animals, except for the occasional bird eating an insect off the sloth (Neam, 2015). Therefore, it is unlikely that sloth symbionts are transmitted from social contact with other sloths or other animals. Sloths of the same species do, however, interact during two phases of sloth life history at which time fur symbionts could be transmitted: mating and early development. Sloths mate with the male on the back of the female or face-to-face, and can copulate for up to seven minutes (Bezerra et al., 2007; Dias et al., 2009; Richard-Hansen & Taube, 1997; S. Trull, unpublished data). Close physical contact during copulation could allow for the transmission of symbionts, especially mobile symbionts, such as arthropods, along with any microbes they might carry. Between the birth of young (gestational

period of 6-10 months), sloths mate every 10-15 months for a total period of ~20 years (Taube et al., 2001); this amounts to approximately 10 matings over the life of a sloth, often with a different partner. The role of sex and the "reproductive microbiome", the microbiome that makes contact with gametes/offspring or the reproductive tract of another organism via mating (Rowe et al., 2020), on the transmission of fur symbionts between sloths is unknown.

Sloths give birth to their young in the canopies of trees, and newborn sloths immediately cling to the fur of the mother sloths' abdomen for a continuous period of six to nine months (Ramirez et al., 2011). Newborn sloths generally cling to the abdomen of their mother, not her back; however, juvenile sloths do climb onto the back and sides of the mother when she is stationary (Soares & Carneiro, 2002; S. Trull, unpublished data). It is not clear what microbes grow on the abdomen of sloths, since all sloth hair microbiome studies to date have sampled from the greenest parts of the sloth, generally the head, shoulder, and back (Pauli et al., 2014; Suutari et al., 2010; M. Kaup and S. Trull, unpublished data). Juvenile sloths remain on their mothers for so many months, therefore, fur microbes/symbionts are likely vertically transmitted due to protracted close contact. At the very least, mothers dictate the exposure of their young to environmental species pools by the nature of their own movement throughout the forest canopy. Microbes are known to play a fundamental role in the development of most animals (McFall-Ngai et al., 2015; Bosch et al., 2019) and this may also be true for sloths.

Sloths spend upwards of 70% of their waking hours resting in trees (Chiarello, 1998; Urbani & Bosque, 2007). They are often in direct contact with tree bark and leaves during their sleeping and resting hours, as they can be routinely found laying on branches or in an upright position, reclining against a branch or the trunk of a tree (S. Trull, unpublished data). Thus, transmission of biota from trees to sloths and vice versa is very likely, although there is little data

to formally support this hypothesis. The phyllosphere is teeming with microorganisms, such as bacteria, archaea, fungi, and algae (Vacher et al., 2016), and with metagenomic tools, one could compare the structure and function of microbial communities on sloths and their surrounding canopy environment (Rastogi et al., 2013; Baldrian, 2017; Hassani et al., 2018). Sloths also interact with soil when they descend to the base of a tree to defecate once a week (Pauli et al., 2014; Voirin et al., 2013), where they could acquire or disperse symbionts. The arthropods that reside in sloth fur may also be vectors that transmit symbionts to and from sloths.

COMPONENTS OF THE SLOTH FUR ECOSYSTEM

1. Algae

The green hue of sloths arises from green algae that grow on sloth hair (Aiello, 1985; Suutari et al., 2010). Cyanobacteria may also contribute to this greenish hue, although only one species, *Oscillatoria pilicola*, has been identified to the species level thus far (Table 2; Wujek & Lincoln, 1988). DNA sequences for red algae have also been found on sloths (Table 2; Suutari et al., 2010). For this chapter, I use the term "algae" to refer broadly to eukaryotic algae and cyanobacteria unless specifically distinguished. It is not clear if algae are resident on all sloths in the wild, which occupy a tropical native range from Guatemala south through Peru and Brazil (Montgomery & Sunquist, 1978) (Figure 2). One study found that 73% of the 74 sampled sloths had visible algae on their fur identified via eye or microscope (*Bradypus variegatus* [n=18], *Bradypus tridactylus* [n=12], *Bradypus pygmaeus* [n=12], *Bradypus torquatus* [n=8], *Choloepus hoffmanni* [n=22], *Choloepus didactylus* [n=2]) (Suutari et al., 2010). However, neither sloth age, season of sampling, nor location were accounted for, and the analysis included captive sloths from zoos, which lack native epibionts (likely due to being bred in captivity, bathed, or

being kept in an enclosed habitat away from potential microbial symbionts in their native habitat). It is also generally overlooked that “brown” sloths may actually host epibiotic algae even though not visibly green to the naked eye (Goffart, 1971): such algae may simply be in a dormant or non-green state when moisture is limited. In fact, wetting of “brown” sloth hair results in a rapid greening within seconds to minutes (Figure 3), akin to what is observed with the wetting of desiccated biological soil crusts (Abed et al., 2014; Pietrasiak, 2014).

Table 2. Known descriptions of algae found in sloth fur. Descriptions derived from Friedl (1995)[a], Printz (1964)[b], Schubert (2003)[c], Suutari et al. (2010)[d], Wujek & Timpano (1986)[e], or otherwise AlgaeBase.org (Guiry & Guiry, 2019).

Genus	Phylum	Class	Description
Trichophilus	Chlorophyta	Ulvophyceae	small (3-13 μm) thick-walled cells with numerous, small, discoid chloroplasts that lack pyrenoids[b,d]
Trentepohlia	Chlorophyta	Ulvophyceae	filamentous, orange in color
Pseudendoclonium	Chlorophyta	Ulvophyceae	filamentous, marine, cells with single parietal chloroplast and a pyrenoid
Trichosarcina	Chlorophyta	Ulvophyceae	filamentous, cells with single parietal chloroplast and pyrenoid
Ulothrix	Chlorophyta	Ulvophyceae	unbranched filaments with cells always closely adherent, uninucleated cylindrical cells
Printzina	Chlorophyta	Ulvophyceae	filamentous, uninucleated cells, chloroplasts parietal and band-shaped
Collinsiella	Chlorophyta	Ulvophyceae	gelatinous, uninucleated cells, cup-shaped chloroplasts
Asterochloris	Chlorophyta	Trebouxiophyceae	found in association with fungus in lichen, single asteroid chloroplast in a crenulate, echinate, or lobed form
Chlorella	Chlorophyta	Trebouxiophyceae	cells spherical, subspherical or ellipsoid, single or forming colonies, chloroplast single, parietal, pyrenoid present
Nannochloris	Chlorophyta	Trebouxiophyceae	subspherical to subcylindrical, 0.8 – 4.5 μm in diameter unicells. May occur in pairs enclosed in mucilage, or in large numbers in a mucilage mass[c]
Trebouxia	Chlorophyta	Trebouxiophyceae	found in association with fungus in lichen, pyrenoid present
Stichococcus	Chlorophyta	Trebouxiophyceae	unbranched filaments, cell walls thin, without gelatinous sheath, cells cylindrical and elongate,

			sometimes slightly oval
Myrmecia	Chlorophyta	Trebouxiophyceae	coccoid cells, found in association with lichenous fungi; not to be confused with the genus of ants by the same name[a]
Dictyococcus	Chlorophyta	Chlorophyceae	zoospores with a single parietal plastid nearly closed and lacks a pyrenoid, spherical cells[e]
Chlorococcum	Chlorophyta	Chlorophyceae	uninucleated cells, ellipsoidal to spherical and vary in size, cell walls smooth, parietal chloroplast and with one or more pyrenoids
Planophila	Chlorophyta	Chlorophyceae	uninucleated cells, spherical, solitary or tightly grouped in small (usually 2–8 cellular) colonies, thin cell walls
Oscillatoria	Cyanobacteria	Cyanophyceae	Filamentous, trichomes blue-green to brownish-green, highly motile
Nostoc	Cyanobacteria	Cyanophyceae	filamentous-thallose, gelatinous, cells cylindrical, barrel-shaped up to almost spherical
Fischerella	Cyanobacteria	Cyanophyceae	filamentous-thallose, thallus usually felt-like, usually barreliform cells
Rufusia	Rhodophyta	Stylonematophyceae	branched-filamentous, several parietal, discoidal to band-shaped plastids with no pyrenoid, reddish to violet in color

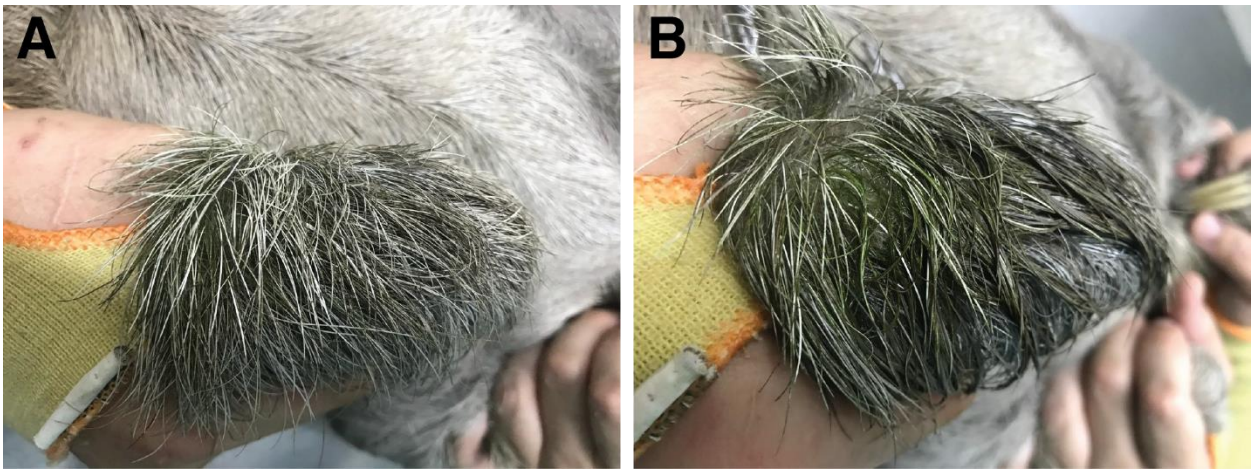


Figure 3. Dry and wet sloth hair. Hair on the back of the hand of (A) a dry *Bradypus variegatus* (brown-throated three-fingered) sloth, and (B) the same hand 10 seconds after wetting reveals a rapid greening and the presence of visually cryptic green algae/cyanobacteria.

a. Sloth Hair Structure and Algal Growth. The morphology of sloth hair has the potential to influence the extent and composition of symbiotic growth. Three-fingered sloth hair has transverse cracks that increase in quantity and depth as sloths age (Figure 4; Aiello, 1985; Wujek & Cocuzza, 1986). The hairs swell considerably when wet, and it has been hypothesized that moisture that is retained within cracks sustains algal growth on the surface of the hairs (Aiello, 1985). It does not appear that the algae grow within the cracks, which would potentially limit access to photosynthetic radiation (Aiello, 1985). It remains unknown whether algae directly colonize hair with very narrow cracks or if they contribute to hair crack development. In contrast, two-fingered sloth hair has vertical grooves and does not absorb as much water; algae appear only to be found within the grooves instead of coating the entire hair (Figure 4B; Aiello, 1985; Wujek & Cocuzza, 1986). Differences in hair architecture may be responsible for the observed differences in fur microbiome surveys between the two genera of sloths (Aiello, 1985; Sutaari et al., 2010). Although increased absorptive properties due to unusual hair structure are not limited to sloths (Kingdon et al., 2012), the unique cracked/grooved hair structure of sloths

seems to facilitate symbiotic algal growth unlike any other mammal (Aiello, 1985). It is unknown whether algal and fungal species typically found on sloth hair are able to grow on texturally smooth hair. Whether such hair cracks/grooves co-evolved with the associated microbes remains an open question. Future research should determine if there is coevolution of traits between sloths and their fur algae, if composition of the sloth-hair microbiome changes as the hair cracks develop and deepen with age, and if this in turn impacts the aging sloth.

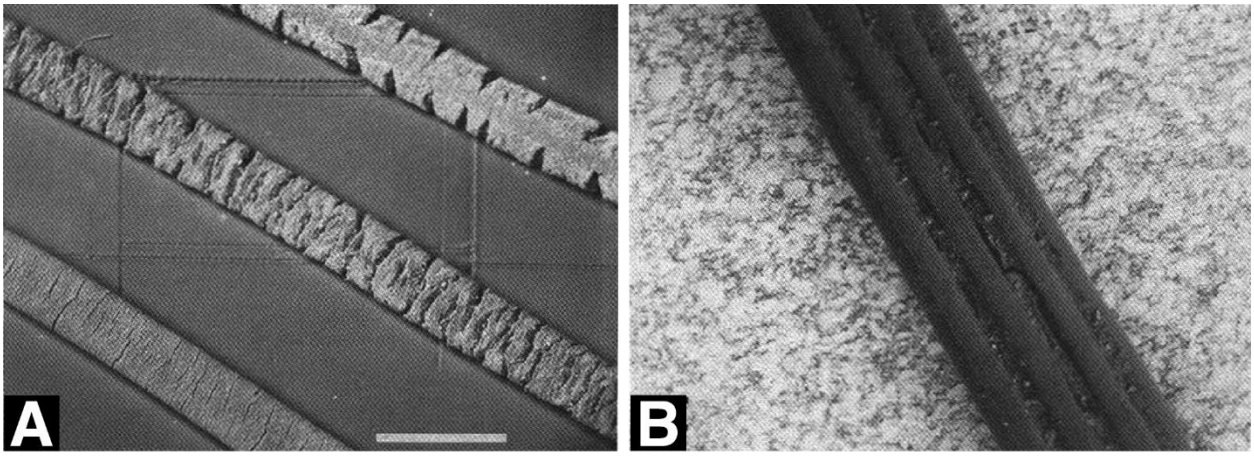


Figure 4. Scanning electron micrographs of sloth hairs. (A) *Bradypus variegatus* (brown-throated three-fingered sloth) hair at three different stages of development (bar = 0.6 mm). The bottom hair is from a young sloth in which transverse cracks are only beginning to develop. The middle hair is from an adult sloth displaying larger cracks. The top hair is from an old sloth and shows deep transverse cracks. (B) *Choloepus hoffmanni* (Hoffmann's two-fingered sloth) hair showing longitudinal ribs or grooves, at 6X higher magnification than in panel A. Photos reproduced from Aiello (1985) (Smithsonian Institution Press).

b. Identification of Sloth Algae. Morphological identification of sloth algae has yielded confusing results; for most cases, the sloth species from which algae have been derived has not been recorded (Table 2). *Trichophilus welckeri*, the most well known of sloth green algae, is one exception, however, and was first identified on sloths in 1887 (Weber-van Bosse, 1887). *Trichophilus* is in the class Ulvophyceae and is characterized by small (3-13 μm) thick-walled cells with numerous, small, discoid chloroplasts that lack pyrenoids (Figure 5; Table 2; Printz,

1964; Suutari et al., 2010). The diversity of green algae and cyanobacteria may be far greater than is suggested by recent studies that focus on *T. welckeri*, and its role in the sloth hair ecosystem (Pauli et al., 2014). Other species of algae should be taken into consideration to properly understand how the community of photobionts is functioning and impacting its accompanying fungal and bacterial symbionts, arthropods, and the sloth itself.

In a conference abstract by Thompson (1972), many sloth fur-associated algae and cyanobacteria were listed, identified solely via morphology. However, algal and cyanobacterial species can be highly similar morphologically, and DNA- and polyphasic-based methods are typically required to make clear taxonomic assignments (Leliaert et al., 2014; Willmotte et al., 2017). Unfortunately, no follow-up confirmations of Thompson's (1972) identifications exist in the literature and Thompson did not specify from which specific sloth species these algae were obtained. Thompson identified two species of *Oscillatoria* and one of *Nostoc*, but it is not clear if either of these *Oscillatoria* are the same as the *Oscillatoria pilicola* identified and described by Wujek and Lincoln (1988) on both the fur of three-fingered *B. variegatus* and two-fingered *C. hoffmanni*. The genus *Fischerella*, three coccoid green algae (including *Dictyococcus bradypodis* and *Chlorococcum choloepodis*), three species of *Trentepohlia*, two of *Stichococcus*, and one of *Nannochloris* were identified (Table 2; Thompson, 1972; Wujek & Timpano, 1986). *Rufusia*, a red algae named by Wujek and Timpano (1986), was identified on both three-fingered *B. variegatus* and two-fingered *C. hoffmanni*.

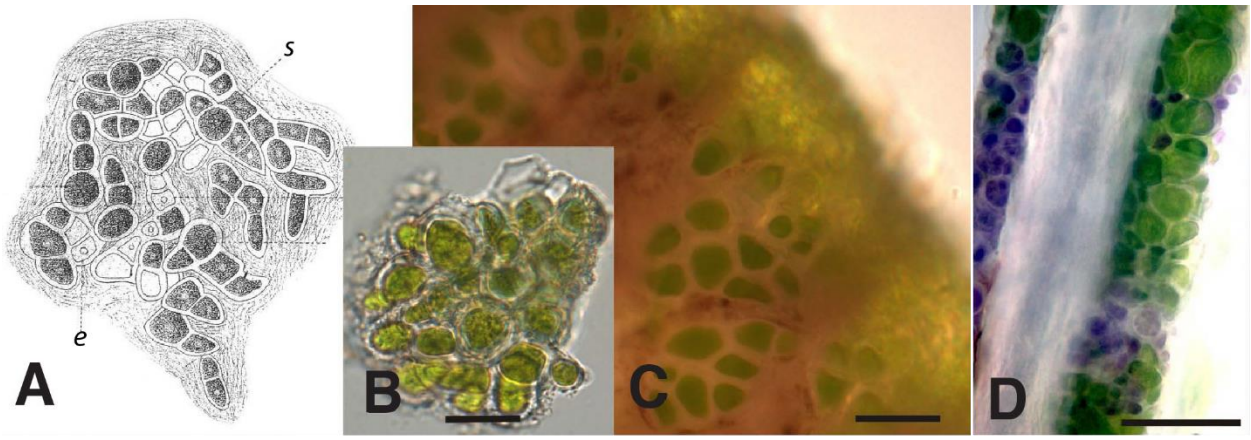


Figure 5. Morphology of green algal clusters, presumably of *Trichophilus welckeri*, found in sloth hair. (A) *Trichophilus welckeri* "fronds" as described by Weber-van Bosse (1887, Fig. 15); "s" refer to sporangia and "e" to empty sporangial cells. (B) and (C) *Trichophilus*-like alga from a hair of the pygmy three-fingered sloth, *Bradypus pygmaeus*. (D) Hair with *Trichophilus*-like alga from a Hoffmann's two-fingered sloth, *Choloepus hoffmanni*. Modified from figure by Suutari et al. (2010; BioMed Central).

Metagenomic studies of sloth fur to date reveal a diverse and variable array of algae across and within different sloth species. The iconic *T. welckeri* was identified using metagenomic techniques in the fur of *B. variegatus*, the pale-throated sloth, *Bradypus tridactylus*, and the pygmy three-fingered sloth, *Bradypus pygmaeus*; to date, no other green algal species have been found on these sloths using 18S amplicon sequencing (Table 3; Suutari et al., 2010). *T. welckeri* has also not yet been found environmentally (Suutari et al., 2010), although this may be a consequence of insufficient environmental sampling across the sloths' geographical range and within the canopies of trees. The maned three-fingered sloth, *Bradypus torquatus*, hosts a variety of algae belonging to genera known to be terrestrial, e.g. *Trentepholia* and *Myrmecia* (Table 3; Suutari et al., 2010). Hoffmann's two-fingered sloth, *Choloepus hoffmanni*, and *B. tridactylus* host the unique genus *Trichophilus* as well as terrestrial green algae from their surroundings (Table 3; Suutari et al., 2010).

The 18S sequences for *Trichophilus spp.* found in association with *B. variegatus*, *B. pygmaeus* and *B. tridactylus* were found to cluster separately from *Trichophilus* sequences obtained from *C. hoffmanni* (Suutari et al., 2010). *Trichophilus spp*. from *Bradypus* and *Choloepus* differ in cell size, and *B. variegatus* and *T. welckeri* phylogenies are consistent with codivergence, which has led some to propose that *B. variegatus* and *T. welckeri* have coevolved (Fountain et al., 2017; Suutari et al., 2010). However, matching phylogenies is an insufficient demonstration of reciprocal coevolution (Janzen, 1980; Anderson, 2015). The differences in hair structure as discussed earlier may impact differential colonization of sloth hair and the poorly charted biogeography of environmental sources of sloth algae might explain the underlying phylogenetic concordance. Future efforts should focus on: (i) further sampling for environmental sources of *T. welckeri*; (ii) identifying coevolved traits/genes and potential reciprocal selection on those traits/genes; and (iii) demonstrating how specific genetic changes within host and symbiont could have occurred as a result of the interaction.

Table 3. Sloth species and associated algal symbionts identified to date. Those with an asterisk following the genus have thus far only been found on sloths and not yet on other environmental substrates. Data is from Suutari et al. (2010; as clarified through personal correspondence with M. Suutari and J. Blomster). Cyanobacteria are indicated by a superscript [C]. Eleven genera not listed in the table, *Chlorococcum, Collinsiella, Dictyococcus, Fischerella*[C], *Nannochloris, Nostoc*[C], *Planophila, Pseudendoclonium, Stichococcus, Trichosarcina,* and *Ulothrix*, were found on sloths, but are of an unidentified origin (Thompson, 1972; Wujek & Timpano, 1986). Note that *Myrmecia* is a genus of green algae associated with lichens.

Sloth Common Name	**Scientific Name**	**Algal Genera**
Brown-throated three-fingered sloth	*B. variegatus*	*Trichophilus*, Oscillatoria*[C], *Rufusia*
Pygmy three-fingered sloth	*B. pygmaeus*	*Trichophilus**
Pale-throated three-fingered sloth	*B. tridactylus*	*Trichophilus**
Maned three-fingered sloth	*B. torquatus*	*Trentepohlia, Myrmecia, Asterochloris, Chlorella, Printzina, Trebouxia*
Hoffmann's two-fingered sloth	*C. hoffmanni*	*Trichophilus*, Oscillatoria*[C], *Rufusia, Trentepohlia*
Linnaeus's two-fingered sloth	*C. didactylus*	No Data

c. Algal Benefits. Several hypotheses have been proposed for how algae might benefit sloths, however, they all lack concrete empirical support, and in fact, it is not clear if the algae provide any benefit to the sloth. It is possible that it is simply a commensal relationship, and that sloths have so much algae in their fur because they do not have the means to clean themselves. Despite this, it is widely believed that fur algae provide a camouflage benefit to the sloth (Aiello, 1985; Pauli et al., 2014; Suutari et al., 2010), but no studies have been pursued to test this

hypothesis. As discussed previously, sloth fur coloration can change: they are primarily green during the rainy season when their hair is regularly wet (Figure 6A), and in the dry season, many sloths lose their greenish hue and appear brown or grey (Britton, 1941; Gilmore et al., 2001). Direct observations of brown/grey sloths in their native canopy suggest that they are very well camouflaged with this color scheme, blending in with the branches, trunks, and dead leaves of trees (Figure 6C & D), as well as resembling ant and termite nests (Figure 6B; Goffart, 1971). It is not known whether sloths' predators use color vision to detect prey. While some predators of sloths, such as eagles, see in color and may be able to differentiate between "green" and "brown," others, such as ocelots and owls that hunt at night, may not. The fact that sloths move slowly and very little could prevent predator detection and aid in their camouflage without the need for green algal growth.

Because of the difficulty of observing predation behavior under natural circumstances, clay models are often used (Bateman, Fleming, & Wolfe, 2017) and sloth clay models could in theory be utilized to understand the effect of sloths' pelage coloration on survival. Practically, sloth camouflage studies may be difficult to perform given: (i) the likely sub-optimal placement of models on small branches within the canopies of trees, which often cannot be reached without a crane; and (ii) the extensive monitoring of models that would be required throughout a rainforest. The lack of movement in sloth models may also be problematic since predators typically detect moving prey much more readily than stationary prey (Paluh, Hantak, & Saporito, 2014).

The relationship between brown-throated three-fingered sloths and Azteca ants that form a mutualism (myrmecophytism) with Cecropia trees, a genus of trees that *B. variegatus* most frequently use for food and refuge, provides another hypothesis for how fur algae might benefit

sloths (Figure 7; Vaughan et al., 2007; Garcés-Restrepo et al., 2019a). Azteca ants fiercely defend these trees from herbivores such as leaf-cutter ants (Schupp, 1986). While it is unknown whether these ants are effective at preventing sloths from eating the leaves of the Cecropia (Figure 7D), anecdotal evidence suggests that sloths are unfazed by these notoriously aggressive biting ants (S. Trull & P. Marting, unpublished data). Given the broad precedence of microbial volatile organic compounds (mVOCs) that deter or modulate insect behavior (Davis et al., 2013; Engl & Kaltenpoth, 2018), it is possible that semiochemicals produced by the microbiota of sloth hair act to repel Azteca ants. While mVOCs from plants (Leach et al., 2017), bacteria, and fungi (Dickschat, 2017; Lemfack et al., 2017) have been investigated, the capacity for algae to produce such compounds has been little explored (Achyuthan et al., 2017; Lemfack et al., 2017). Given the prevalence of bacteria (e.g., *Streptomyces* and *Myxobacteria* (Veselova et al., 2019)) that produce mVOCs in addition to other diverse compounds (Audrain et al., 2015; Lemfack et al., 2017), the omission of a sloth hair bacteria study, and the unexplored algal mVOCs, the sloth fur microbiome may be a reservoir for novel mVOC-producing microbes.

Figure 6. Color and shape similarities of sloths. (A) A female *Bradypus variegatus* (brown-throated three-fingered sloth) with green fur coloration, taken during the wet season; (B) an Azteca ant carton nest that looks similar to a hanging sloth; (C) a dry *B. variegatus* sloth and (D) a dry *Choloepus hoffmanni* (Hoffmann's two-fingered) sloth with similar coloration as the branches, vines, and bark of the trees they inhabit. Photo of Azteca ant nest by Solar (2014) used with permission under Creative Commons License CC BY-NC-SA 2.0**.**

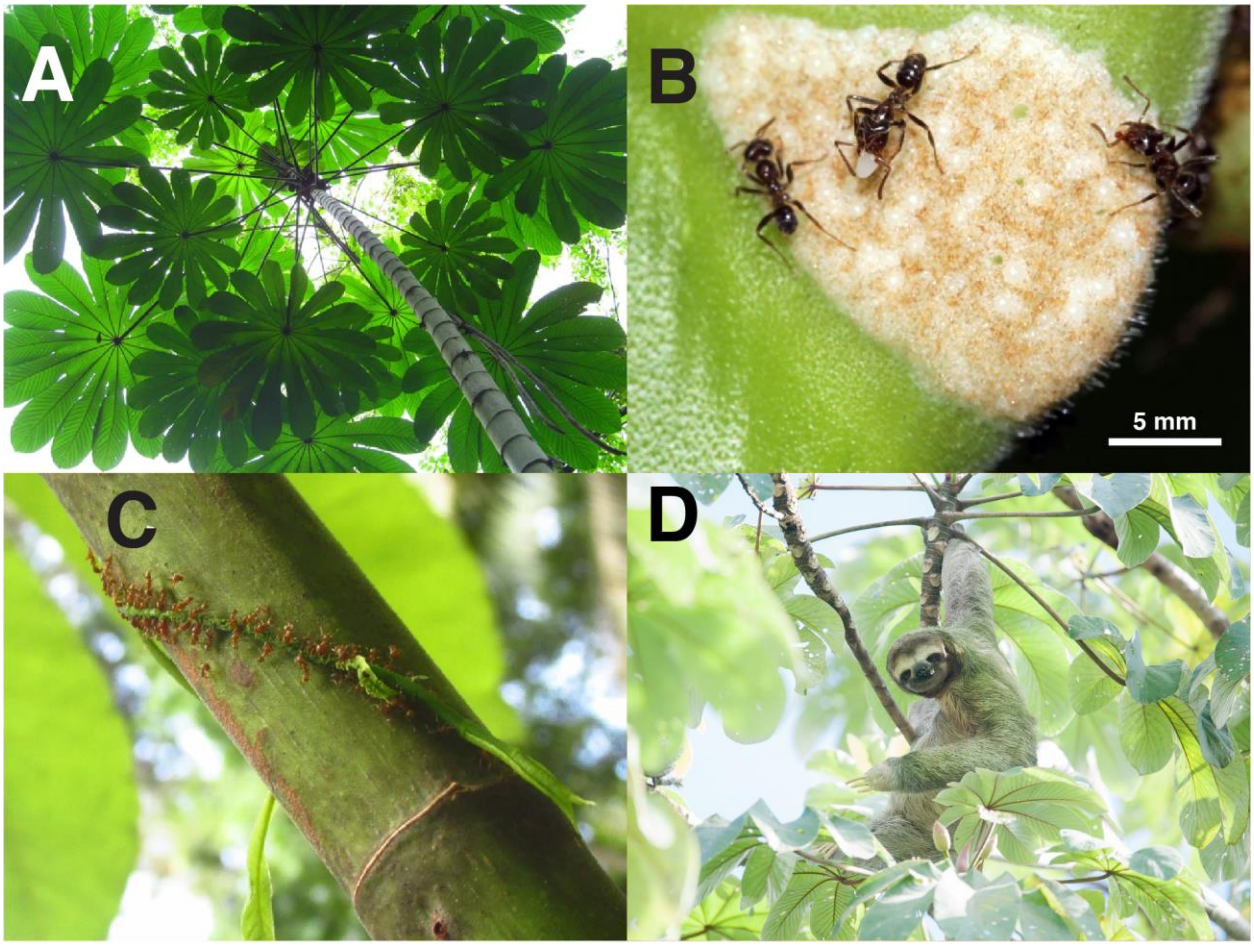


Figure 7. Photographs showing the (A) canopy of a *Cecropia obtusifolia* tree, (B) mutualistic ants, *Azteca constructor*, harvesting food bodies from a *Cecropia* petiole/stalk (bar = 5 mm), (C) Azteca ants attacking an encroaching vine to protect a Cecropia tree, and (D) a Brown-throated three fingered sloth, *Bradypus variegatus*, eating fruit from a Cecropia tree, seemingly unbothered by ants. Panel A, B, and C photos reproduced from Marting et al. (2018) with permission under Creative Commons License CC BY 4.0.

Other proposed hypotheses for how algae could benefit sloths include: (i) algae serving as a nutritional food source (Pauli et al., 2014); (ii) algae being a source of thermal insulation (Aiello et al., 1985); (iii) algae providing some yet unidentified chemical benefit to overall sloth health (Aiello et al., 1985); (iv) algae facilitating beneficial bacterial growth (Suutari et al., 2010); and (v) algae acting as a sunscreen (Suutari et al., 2010). Owing to a limited gut size and a diet of leaves with little nutritional value, *B. variegatus* has been hypothesized to consume the

green algae growing on their fur as a source of nutrition (Pauli et al., 2014). While remnants of green algal cells have been found in their stomach contents (Pauli et al., 2014), this hypothesis lacks evidence (See "Sloth Moths" section below). Another hypothesis suggests that algae may aid in thermal insulation because sloths have difficulty maintaining an even body temperature, although no clear mechanistic or physiological model has been proposed for how such insulation might work (Aiello, 1985; Britton & Atkinson, 1938; Goffart, 1971; Montgomery & Sunquist, 1978). It has been speculated that chemicals produced by fur algae may diffuse along hairs to the skin surface and be absorbed through the skin of the sloth to provide some health benefit (Aiello, 1985). It has also been suggested that sloth algae may produce exopolymeric substances that facilitate beneficial bacterial growth (Suutari et al., 2010). Lastly, *T. welckeri* has been found to produce a UV-absorbing mycosporine-like amino acid, which presumably acts like a sunscreen in shielding sloths from UV radiation (Karsten et al., 2005). These ideas have largely gone untested but the observations beg several general questions. Future research should strive to determine why some sloths have algae while others appear to have little to none, if seasonal variations or algal dormancy matter, if sloth algal diversity or abundance matter, what the function of algae in the sloth ecosystem is, and to what degree the sloth-algae symbiosis is mutualistic and a predictive correlate of sloth health vs. an opportunistic commensalism.

2. Arthropods

a. Biting Arthropods. Sloths are also hosts to a wide range of arthropods living in their fur including parasitic, bloodsucking and biting arthropods such as mosquitoes and sandflies, triatomine bugs, lice, mites, and ticks (Gilmore et al., 2001). Six species of ticks have been found on two- and three-fingered sloths, all from the genus *Ambylomma*, but only two species,

Ambylomma geayi and *Ambylomma varium*, appear specialized for living on sloths as these ticks are rarely found on other hosts (Waage & Best, 1985). Tick infestation can be extremely high. At the Instituto Nacional de Pesquisas da Amazonia in Manaus (Brazil), 99% of three-toed and 86.7% of two-toed sloths carried *Ambylomma spp.* (Waage & Best, 1985). Nothing is known about how *A. geayi* or *A. varium* find a host sloth and no correlation has been found between the numbers of ticks at any life stage on a sloth or seasonal differences in rainfall (Gilmore et al., 2001). The blood-sucking mites, *Liponissus inheringi*, *Lobalges trouessarti*, and *Edentalges bradypus*, have been identified on three-toed sloths (Waage & Best, 1985) and the mite *Edentalges choloepi* has been found on Linnaeus's two-fingered sloth, *Choloepus didactylus* (Fain, 1964). It remains an open question how the sloths' ectoparasite loads correspond with sloth health.

b. Commensals and Beetles. Many commensal arthropods are found in association with these slow-moving mammals. It is quite possible that the algae on sloth fur serves as a food source for these commensal arthropods considering that mites and other insects display algophagy (Seniczak, 2016; Mckenna et al., 2015). Cockroaches have been found in sloth fur (Britton, 1941), although this may be quite rare (S. Trull, unpublished data). Adults of several scarab beetle species are frequently found in the fur of three-fingered sloths (of which the beetle in Figure 8 is an example), but have not been reported to be associated with *Choloepus* (Gilmore et al., 2001; Ratcliffe, 1980). The scarab beetles occur near the elbow or on the flanks behind the knees, buried deep inside the fur. The beetles found living on sloths are considered commensal because they are phoretic coprophages: the beetle larvae (and possibly adults) feed on sloth dung and they don't appear to harm the sloths (Gilmore et al., 2001; Ratcliffe, 1980). About a

thousand of such beetles (*Trichillum adisi*) have been found in the fur of a single brown-throated three-fingered sloth (*B. variegatus*) collected on Curari Island in the Central Amazon region (Waage & Best, 1985). Beetles of the genus *Uroxys* have been recorded from sloths in Bolivia, Brazil, Colombia and Panama (Waage & Best, 1985). Despite the ubiquity of beetle-sloth interactions, little is known about the dispersal and density fluctuations of these beetles on sloths, although in Panama, there seem to be higher numbers of beetles during the rainy season (Wolda & Estribi, 1985). It has been suggested that the beetles have dispersal flights at the beginning and end of the rainy season and that part of the population might enter reproductive diapause and disperse from the sloths to sites with some moisture; they presumably resume reproduction at the end of the dry season and return to the sloths (Wolda & Estribi, 1985). Just as there is no data to substantiate an effect of parasite load on sloths, no analysis has been performed to understand the effect of these suspected commensal arthropods or of total arthropod load on sloth health. Likewise, little is known of the potential role these beetles might play in the ecosystem. It is possible that beetles contribute to parasite suppression, secondary seed dispersal, and to nutrient cycling within the sloth fur ecosystem and the larger forest ecosystem (Nichols et al., 2008). It is also possible that some sloth-associated arthropods play a protective and mutualistic role by preying on ectoparasites in sloth fur (cf. Ostlund-Nilsson et al.; Goedknegt et al., 2012).

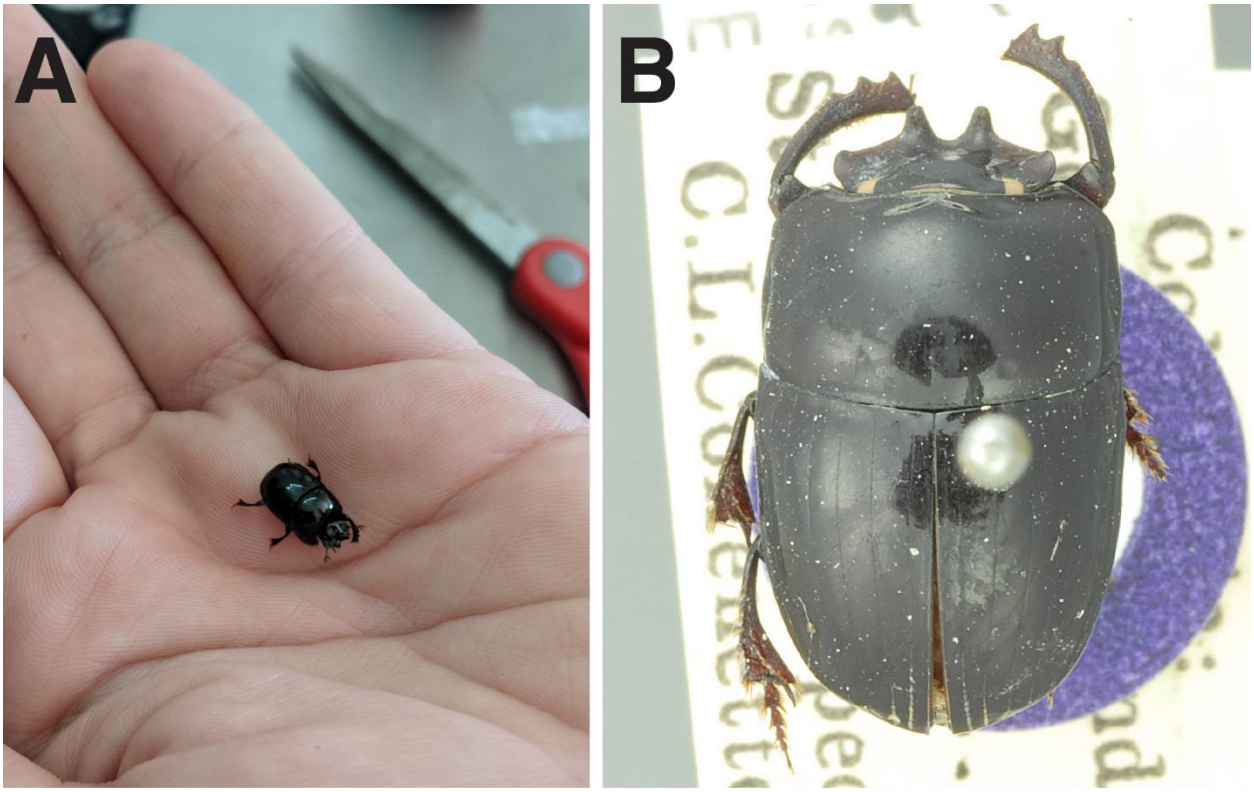


Figure 8. The sloth-associated scarab beetle "*Uroxys gorgon* Arrow, 1933." (A) Collected live from the fur of a *Bradypus variegatus* (brown-throated three-fingered) sloth, and (B) a mounted specimen (Larsen, date unknown), used with permission under Creative Commons License CC BY-NC 3.0.

c. Sloth Moths. Sloth moths in the genus *Cryptoses* have received notable attention as a sloth symbiont. There is appreciable geographic sympatry amongst sloth-associated moth species and several different species may coexist in the fur of a single sloth (Waage & Best, 1985). Different sloth moth species appear to be found on all species of sloths (Bradley, 1982; Pauli et al., 2014; Waage & Best, 1985). *Cryptoses choloepi* seems to be the dominant moth found on *B. variegatus* and has been studied almost exclusively in relation to this sloth species (Figure 9). Female *C. choloepi* moths that live in *B. variegatus* fur have been observed to oviposit in the dung of the sloth as the sloth descends to the forest floor to defecate, about once a week. Moth larvae in early stages spin silken threads between 2-3 pellets of dung, forming net-like structures

from which they feed (Waage & Montgomery, 1976). Upon maturation, newly emerged moths fly from the dung pile into the forest canopy to find a new sloth host (Waage & Montgomery, 1976). In addition to nutritional benefits the sloth moth larvae presumably receive from feeding on sloth dung, it is possible that adult moths eat sloth/algal secretions or hair-associated microbes (Figure 9). The sloth moth gut microbiome has yet to be explored, which may provide evidence for this. Adult moths are believed to receive a transportation benefit as well as a protection benefit from living in sloth fur (Waage & Montgomery, 1976; Wolda, 1985). However, the amount of protection moths receive in association with sloths is questionable, since brown jays have been observed to predate insects off sloth fur (Neam, 2015).

Figure 9. The sloth moth, *Cryptoses choloepi*, on a *Bradypus variegatus* (brown-throated three-fingered) sloth. (A) Moths often swarm the sloth's face, especially orifices such as the nose and eyes, and (B) appear well camouflaged on the sloth's grey-brown fur.

Based on studies to date, it would appear that sloth moths have a commensal relationship with their sloth hosts. However, a three-way mutualism has been proposed involving *B. variegatus*, their moths, and fur algae, particularly *T. welckeri*. According to this hypothesis,

moths are portals for nutrients, increasing nitrogen levels in sloth fur through defecation, which is believed to promote algal growth (Pauli et al., 2014). *T. welckeri*-like algae have been found (microscopically) in sloths' stomach contents, which has led to the hypothesis that sloths consume these algae to augment their limited diet. With this set of observations, the proposal is that sloths are involved in an evolutionary trade-off in which they risk their lives, descending to the ground to defecate, in order to preserve this sloth-moth-algae tripartite mutualism (Pauli et al., 2014). There are five potential problems with this hypothesis. First, morphological designations of algal species are not a definitive method to identify species, especially given how this taxon is often morphologically cryptic and under-studied in general (Dudgeon et al., 2017). Second, while the main groups of bacteria that inhabit the gut microbiome of *B. variegatus* have been identified (Dill-McFarland et al., 2016), no metagenomic studies to date have been performed to characterize the eukaryotic diversity in this species' gastrointestinal tract. Sloths have, however, been observed licking and eating material off of branches and tree trunks, which may include lichens (Tirler, 1966; S. Trull, unpublished data). Due to limited sampling, it is not clear if algae found on sloths may also grow on leaves and bark in tree canopies, and thus sloths may be eating algae from their environment, not from their fur. Third, thousands of hours of sloth behavioral research recorded during the day and night do not support the idea that sloths lick themselves (like cats) or eat epibiotic algae from their fur (Tirler, 1966; S. Trull, unpublished data). Fourth, only two *B. variegatus* individuals out of twelve sampled in one location in Costa Rica were identified as having *Trichophilus spp.* in their stomachs (Pauli et al., 2014). And lastly, if sloth tree-descent and ground-defecation is driven by a need to benefit moths via dung oviposition, one would expect there to be reciprocal fitness benefits provided to the sloth by the moths in order for this behavior to have evolved or be maintained (Voirin et al.,

2013); however, the implied and indirect benefits that sloths might obtain from moth-influenced fur algal growth may be quantitatively modest and lack empirical support.

Many ideas have been proposed to explain the sloths' unusual defecation behavior, which, with evidence, could disprove or complicate this three-way mutualism. It has been proposed that defecating on the ground (as opposed to letting dung drop from the canopy of trees) is a strategy that sloths use to go undetected, since being quiet and hidden seems to be their predominant life strategy and defecating from the canopies of trees presumably may cause a disturbance that attracts predators (S. Trull, unpublished data). However, there is no evidence that descending to the base of the tree is risky to the sloth, especially since the majority of their predators, harpy eagles, spectacled owls, ocelots, and tayra, can also detect and attack them from the tree canopy, often by knocking them to the ground where they proceed to eat them (Voirin et al., 2009; Izor, 1985; Bezerra et al., 2009; Beebe, 1926). Other theories include proposed benefits from fertilizing their most frequently used trees, communicating with other sloths through social latrines, trying to hide their scent from predators, or deriving nutritional benefits from consuming soil while on the ground (Beebe, 1926; Krieg, 1939; Goffart, 1971; Voirin et al., 2013). Observational data suggests that three-fingered sloths do not frequently eat soil (S. Trull, unpublished data) and no data exist in support of the other theories. Regardless, to determine if symbiotic moths somehow benefit the sloth, directly or indirectly, or if it is simply a commensalism, requires more careful, empirically driven studies of the nature and benefits associated with this sloth-arthropod-microbe fur ecosystem.

3. Fungi

Fungi are known to be associated with sloth hair, but the roles they might play in the community ecology of the sloth pelage and in the health of the sloth remain unexplored. A diverse group of Ascomycota and one Basidiomycete (*Sporobolomyces subbrunneus*) have been identified growing on sloth fur through sequencing and culture-based methods (Suutari et al., 2010; Higginbotham et al., 2014). Only two species of fungi that have been found on sloths have also been found on the bark of trees in sloth habitats (*Devriesia staurophora* and *Mycosphaerella pini*; Suutari et al., 2010), although these results are from very limited sampling. These sloth-associated fungi have been found in soil and plants (Arnold and Lutzoni, 2007; Wang et al., 2011), so it is possible that the sloths are exposed to these fungi when they defecate on the ground or as they eat and interact with leaves and bark (Higginbotham et al., 2014). Nearly 35% of fungal isolates obtained from *B. variegatus* fur are identical to endophyte strains obtained from plants (Higginbotham et al., 2014). Given the taxonomic similarity between endolichenic and endophytic plant fungi in the same environments (U'ren et al., 2012), it seems plausible that some sloth hair fungi may associate directly with green algae (Higginbotham et al., 2014). Previous studies support that fungi, and these taxa in particular, have intrinsic affinities for associating and forming mutualisms with algae, as seen in lichens (Hawksworth, 1988; Arnold et al., 2009) and other systems (Hawksworth, 2000; Gareth Jones et al., 2012; Hom & Murray, 2014; Du et al., 2019), but direct tests of these sloth fungal taxa with algae need to be conducted to confirm whether they form mutualisms or not.

Whether these fungi are commensals or are parasitic or mutualistic is not clear, but certain species may be beneficial to humans, and thus may similarly benefit sloths. Hair-associated fungi from *B. variegatus* have been shown to display a broad range of inhibitory

activities against parasites that cause malaria (*Plasmodium falciparum*) and Chagas disease (*Trypanosoma cruzi*), human breast cancer cells, and bacteria, particularly Gram-negative bacteria (Higginbotham et al., 2014). Some sloths have clear black fungal growth on their hair (Figure 10A), which could potentially harm the sloth or outcompete other microbes in the sloth hair ecosystem. Others develop severe fungal infections on their skin that can be detrimental because the infections produce scabs, which then fall off, leaving bare skin that is susceptible to parasites like ticks and mosquitos (Figure 10C); anecdotally, fungal infections generally correlate with sick sloths (S. Trull, unpublished data). Many questions remain regarding these parasitic fungi, such as what causes or triggers these fungal infections, and are these fungi externally acquired or are pathogens resident and dormant and then become activated? Because of the plasticity of symbiotic interactions and the potential for mutualists to switch to parasites (Akçay, 2017; Jones et al., 2015; Kogel, Franken, & Hückelhoven, 2006; Leung & Poulin, 2008; Vostinar & Ostria, 2019), it is entirely possible that these fungi are normally commensal or mutualistic with sloths but become pathogenic due to environmental shifts or microbiome imbalances/dysbiosis.

Figure 10. Sloths and fungi. *Top:* the back of the heads of two *Choloepus hoffmanni* (Hoffmann's two-fingered) sloths with visible growth on the fur of (A) black fungi and (B) algae. *Bottom:* facial photos of (C) a *Bradypus variegatus* (brown-throated three-fingered sloth) with a severe fungal infection that causes scabs of hair to fall off, and (D) a healthy *B. variegatus* sloth for comparison.

Some interactions of fungi with sloth algae may resemble that of lichens, which are typically slow growing and commonly found on trees that are undisturbed. Given the slow movements of sloths, which perhaps can be more easily colonized, being more similar to a tree than many fast-moving animals, and the presence of algae in their fur, sloths may be reservoirs of lichenous fungi and lichen-like fungal associations. Epizoic lichens, fungi, and/or cyanobacteria have been found to grow on arthropods, specifically two species of leaf mantis in the genus *Choeradodis* (Lücking et al., 2010) and various harvestmen arachnids (within small pits) (Machado & Vital, 2001; Proud et al., 2012; Young et al., 2018). Fungal-algal associations in sloth fur could potentially link sloths to arthropods and bacteria.

4. Other Symbionts

In addition to algae, arthropods, and fungi that live and thrive within the pelage of sloths, other putative fur-associated organisms have been identified through 18S amplicon sequencing; these include euglenozoans, amoebozoans, cercozoans, apicomplexans, dinoflagellates, and ciliates (Table 4; Suutari et al., 2010). To date, nothing is known about the role of these organisms within the sloth hair ecosystem. Apart from the sloth fur cyanobacteria mentioned above (Table 2), fur-associated prokaryotes have not been well documented or sufficiently taxonomically resolved. Surprisingly, a 16S survey of the bacterial diversity on sloths has not been performed; it will be important to survey the prokaryotes present in the sloth fur ecosystem and to understand the inter-kingdom interactions they may have with the sloth and other fur symbionts. Understanding the bacterial diversity in sloth fur will not only allow us to better comprehend the ecology of sloths' fur symbionts and how they might impact the sloth, but will also make sloths a more relatable model system, given the focus on bacteria in microbiome

studies. Could bacterial symbionts influence the function of sloth-associated fungi and algae, as they do for fungal endophytes associated with plants (Hoffmann & Arnold, 2010; Partida-Martínez & Hertweck, 2005) and lichens (Grube & Berg, 2009; Bates et al., 2011)?

Table 4. Other symbionts found in sloth fur. Species names were assigned based on the closest known matches in GenBank. Percentage similarity is to the closest match in GenBank. Data from Suutari et al. (2010). Given the low similarity for most matches and little taxonomic follow-up, these species designations may not be correct.

Phylum	**Species**	**Percentage Similarity**
Euglenozoa	*Petalomonas cantuscygni*	82%
Amoebozoa	*Lamproderma ovoideum*	85%
Cercozoa	*Cercomonas plasmodialis*	99%
Apicomplexa	*Eimeriidae sp.*	89-99%
Dynophyceae	*Heterocapsaceae*	89-91%
Ciliophora	*Bresslauidea discoideus*	97%
	Campenella umbellaria	87%
	Colepidae sp.	95%
	Epistylis galea	88-93%
	Opercularia microdiscum	87-91%
	Peritrichia sp.	87-91%
	Trithigmostoma steini	90%

Sloths are carriers for a variety of arthropod-associated viruses (arboviruses; e.g., phleboviruses, encephalitis viruses, and Oropouche viruses) as well as insect-born protozoans (e.g., trypanosomes, such as *Leishmania*; Gilmore et al., 2001) which may be in blood and fur, since these arthropods bite sloths, but also interact closely with sloth fur. Phlebotomine sandflies on sloths are known carriers of *Leishmania*, which causes leishmaniasis in humans (Arias & Freitas, 1978; Christensen et al., 1982; Herrer & Christensen, 1980). *C. hoffmanni* sloths likely become infected by the trypanosomes in their first few months of life and remain infected for a long time, but appear asymptomatic and do not show signs of pathology (Herrer & Christensen, 1980).

Sloths have unique gut microbiomes as well that may be dictated by their arboreal folivory (Delsuc et al., 2014; Dill-McFarland et al., 2016). Unlike other mammalian herbivores, the bacterial phyla Proteobacteria and Firmicutes dominate the gut microbiome of sloths, and it has been hypothesized that these gut bacteria are largely non-transient residents (Dill-McFarland et al., 2016). Captive sloths fed more low-fiber pelleted food than what might exist in the wild show a large proportion of bacteria in the phylum Bacteriodetes (Delsuc et al., 2014; Dill-McFarland et al., 2016), suggesting diet-driven plasticity of the sloth gut microbiome. A highly abundant *Neisseria* species (Class Beta-proteobacteria) in particular was found in the gut of wild sloths that may be sloth-specific (Dill-McFarland et al., 2016). It remains to be determined how much of the bacteria found in the sloth gut microbiome overlap with those of the fur microbiome, and whether there is overlap of other taxa (like fungi and algae) as well. Unfortunately, only 16S studies of the sloth gut microbiome have been pursued (Dill-McFarland et al., 2016), so we know little about eukaryotic microbes that might be resident within the gut. Green algal fragments have been identified in the stomach contents of *B. variegattus* (Pauli et al., 2014), although there are problems with the taxonomic identification of these fragments by morphology (as mentioned previously). Green algae may be a transient and rare food item and not a component of the gut microbiome.

FUTURE DIRECTIONS

1. Comparing Microbiomes of Convergently Evolved Hosts

While convergent evolution of microbiomes across various organisms has been studied (Fan et al., 2012; Moeller et al., 2013; Delsuc et al., 2014), sloths provide a unique opportunity to compare microbiomes between hosts (last common ancestor ~27-34 million years) that have

convergently evolved (Delsuc et al., 2019; Presslee et al., 2019). Future research should determine how similar the microbiomes of sloths are vis-à-vis other convergently evolved traits, and to what degree host traits vs. competition/cooperation between microbes and symbionts at higher trophic levels influence community structure and function of the fur ecosystem (cf. Foster et al. 2017). A comparison between two-fingered vs. three-fingered sloths may shed light on the weight of selective factors that influence convergent multispecies interactions—the independent evolution of multispecies interactions with similar physiological or ecological functions (Bittleston et al., 2016; Bittleston et al., 2018). These sloth systems may also yield insights into whether functionally redundant "ecotypes" of microbes (in which specific microbial taxonomic designations may not be important because they perform the same ecosystem function) might be more relevant in describing the microbiome and the impact of environmental and host factors (Fetzer et al., 2015; Doolittle & Booth, 2017; Louca et al., 2018).

The rich fur ecosystem of sloths provides an interesting opportunity to explore the interrelationship between gut and fur microbiota from an evolutionary perspective. Being internal to the animal, the gut microbiome is conceivably more shielded from environmental fluctuations than the fur microbiome and both may ostensibly have different (vertical) transmission dynamics. The degree of vertical transmission of the microbiome/symbionts community in both two-fingered and three-fingered sloths would be critical to determine as it dictates the extent of coevolution with the sloth host.

2. The Sloth Holobiont

It may be advantageous to consider the sloth mobile ecosystem from the point of view of holobiont/hologenome theory or as functional unit subject to selection (Bordenstein & Theis,

2015; Meng et al., 2018; Rosenberg & Zilber-Rosenberg, 2018; Roughgarden et al., 2018; Simon et al., 2019). Determining the degree of vertical vs. horizontal transmission of sloth fur symbionts will help establish whether they could have co-evolved with sloths and to identify aspects of the hologenome theory of evolution that might be applicable to the sloth holobiont (Bordenstein & Theis, 2015; Hester et al., 2015). It is unknown how much of the sloth holobiont community is a result of repeated re-assembly from environmental species pools vs. selected for through generational transmission and coevolution with the sloth host; co-evolution would require high partner fidelity and vertical transmission. Effort should be made to understand the extent to which the sloth fur ecosystem (i) can be described by niche-selective vs. neutral theories of assembly (Hubbell, 2001; Miller et al., 2018), (ii) is "isolated" or selected for to be distinct from a sloth's environment, and (iii) a product of coevolution vs. ecological fitting (Janzen, 1980) vs. random chance assemblages of simply what is readily available from the environment. Empirical studies that monitor the colonization process of a newborn sloth, as well as inventorying the environmental biota in the surrounding tree canopy will clarify how sloths acquire their symbionts and is an important step forward in answering these questions. Efforts should also be made to determine which sloth symbionts (if any) might be obligately dependent and thus more likely to have co-evolved: these species might exert a relatively greater influence on host fitness and fur ecosystem structure (cf. Kopac & Klassen, 2016).

As a mobile ecosystem, sloths could be a model for examining microbial interactions at different hierarchical levels within an expanded "eco-holobiont" framework (Singh et al., 2020) whereby biotic feedbacks between microbes and higher trophic levels ("microbial loop") of an ecosystem are explicitly considered in understanding how host ecosystems are shaped and structured (Seibold, et al., 2018; Liu et al., 2019). This would entail viewing the gut microbiome,

fur ecosystem, and the sloth with its surrounding environment as nested parts of a whole; studying the interrelationships across these domains is likely to be more fruitful than studying each component in isolation. Different taxa and genetically encoded functions may fill particular functional roles within this collective mobile ecosystem. Niche theory (Carmona et al., 2016) and metacommunity theory (Leibold et al., 2004; Miller et al., 2018; Leibold & Chase, 2019) could provide useful multi-scale frameworks for dissecting: (i) the contributions and functions of different taxa, (ii) the functional redundancy that might exist across tiers, and (iii) the role of feedback loops in community structure and function.

3. The Nature and Network of Sloth Symbiont Interactions

Thus far, little has been attempted to simply determine the nature of the interactions between sloths and their fur symbionts. Building upon the knowledge from limited studies, efforts should aim to identify the symbiotic traits of each interacting organism and the selective pressures acting on those traits. The ecosystem functions of sloths within their native habitat are largely unknown, although they are believed to be an important source of long-term, stable nutrients at the base of trees where they defecate (Montgomery & Sunquist, 1975). It will be important to determine through environmental sampling if algae like *T. welckeri* are generally limited to growth on sloths or if they can grow independently on other environmental substrates within the sloth habitat. If found environmentally, it would provide support for a model in which sloths acquire algae from the environment and provide a proper null model by which to assess sloth-algae coevolution. An assortment of other organisms are found in sloth fur, including bacteria, euglenozoans, amoebozoans, cercozoans, and alveolates (Table 4; Suutari et al., 2010; Wujek & Lincoln, 1988), many of which appear not to be found readily in the environment

around sloths (Suutari et al., 2010). The functions of these organisms in the sloth hair ecosystem are unknown but have the potential to directly impact sloth health. Sloths appear to be carriers for several arthropod-borne viruses and parasites and understanding the basis for why sloths seem not to be burdened by such pathogens may be of relevance to human health. Also unclear is the role that microbial symbionts have in facilitating host defence against pathogens in general, which has been well demonstrated in plant and pollinator systems (Liu et al., 2019).

Photoautotrophic algae are at the bottom of the food web in many ecosystems (Brocks et al., 2017; Kohlbach et al., 2016; Segovia et al., 2015; Polis & Hurd, 1995), and they likely serve as the base of the sloth fur ecosystem as well. It is unclear how algal growth influences the composition of the rest of the microbiome and if arthropods farm and/or consume the algae. Microbial symbionts in sloth fur may provide supporting services, including producing 'pioneer' metabolite products that provide a foundation for community development, biofilm formation, nutrient cycling, and a thriving ecosystem (McKenney et al., 2018). As a poorly studied reservoir for potentially novel microbial and genetic diversity, these hair algae/microbes may produce specialized or secondary metabolites that prevent infections or volatiles that repel ectoparasites/predators or attract arthropods in a manner similar to how plants use volatiles to attract or repel pollinators and predators (Kessler & Baldwin, 2001; Pichersky & Gershenzon, 2002). In so doing, these natural products may play a vital role in the chemical ecology of the fur ecosystem and in shaping symbiont community structure. Microbes associated with the insects are known to be a source of bioactive compounds and enzymes that have biotechnological potential (Berasategui et al., 2015) and sloth microbes may ultimately be of relevance to human health and agriculture.

It is becoming evident that explicit consideration of spatial, temporal, and phylogenetic scales (specifically ideas of granularity and extent) along with system nestedness will be critical to elucidating both the patterns and mechanisms of community assembly in ecosystems for which microbes play a foundational role (Addicott et al., 1987; Wiens, 1989; Wang & Loreau, 2014; Shade et al., 2018; Ladau & Eloe-Fadrosh, 2019). The complex nested nature of the sloth-forest ecosystem makes it an attractive system to study using ecological network analysis (Fortuna & Bascompte, 2007; Stouffer et al., 2009; Ivens et al., 2016). Network theory can be used to determine where sloth fur symbionts fall on the continuum of specialist to generalist. Studying sloth-symbiont networks may reveal symmetric or asymmetric specialization in different species interactions (Futuyma & Moreno, 1988; Thompson, 1994; Vázquez & Aizen, 2004), for example, in which a specialist alga (e.g., *T. welckeri*) might interact with a generalist sloth (*B. variegatus*). Whether a specialist alga could be more likely to persist in variable environments (i.e., across the geographic range of *B. variegatus*) because it relies on a more common and stable species (Bascompte et al., 2003; Ashworth et al., 2004; Bastolla et al., 2009) is unknown. Also, whether the sloth fur ecosystem could be a nested network with significant asymmetric specialization, which could minimize competition and increase biodiversity (Bastolla et al., 2009), remains to be tested. Modularity analysis (Olesen et al., 2007) can be used to identify keystone species within the sloth ecosystem and assess potential fragility of the system to anthropogenic change (Bascompte & Stouffer, 2009).

As largely solitary creatures (Soares & Carneiro, 2002; Taube et al., 1999; S. Trull, unpublished data) that share a common forest ecosystem and range, it would be interesting to consider how much of the sloth fur community could be understood from the perspective of island biogeography (MacArthur & Wilson, 1967; Bell et al., 2005; Peay et al., 2007; Wilson,

2010; Belisle et al., 2012; Glassman et al., 2017; Proctor & Relman, 2017). The sloth may be a good model system for testing metacommunity theories about feedbacks and species pools; for example, to understand how different communities within "patches" of fur on different sloths (or even at different locations on a single sloth; cf. Proctor & Relman, 2017) are influenced by feedbacks between environmental species pools and the sloth host (Miller et al., 2018). To do this, it will be critical to map potential species pools from the environment and their modes of dispersal, and identify host specific behaviors that influence holobiont composition. To date, the taxonomic richness within the sloth pelage and of species dispersal into and out of the sloth fur remains poorly characterized. It is possible that the sloth arthropods that colonize sloth fur are vectors/dispersers of algae and other microorganisms that thus far have no apparent source in the immediate surroundings of the sloth.

4. Access to a Unique Ecological Regime in Time and Space

I have referred to sloths as a "mobile ecosystem" to highlight the fact that sloths experience life and movement within an unusual regime of time and space, unlike most other macro-organisms. The slow movements of sloths through their geographical range and the vertical column of the forest canopy may allow us to examine an ecological and spatiotemporal regime not typically accessed by sessile (e.g., plants/trees) or significantly more mobile organisms of comparable size. Sloths may provide unique insights into ecological connectivity and movement ecology of wild, free-ranging animals (cf. Jacoby & Freeman, 2016). As discussed earlier, sloths can travel ≥38 m per day and be found at various vertical heights between the forest canopy and the ground, to which they descend once a week to defecate. The abundance and diversity of microbes and arthropods that take up residence within the sloth

pelage begs the question as to whether the uniquely slow timescales at which sloths move, coupled with their vertical migration, might facilitate this phenomenon. Perhaps there is some sort of temporal resonance of ecosystem processes with sloth movement dynamics that facilitates the striking biodiversity on sloth fur. Future studies should determine how community diversity changes as a function of the characteristic timescales of underlying assembly/dispersal processes and if this can be predicted using metacommunity theory.

The recent advances in GPS tracking and remote-sensing/monitoring technology (Kays et al., 2015; Lennox et al. 2017; Neethirajaran, 2017; Taylor et al., 2017; Hughey et al., 2018; Shipley et al., 2018; Ripperger et al., 2019; Williams et al., 2019) will facilitate data acquisition to answer questions of movement ecology, symbiont transmission, and context-dependency of the sloth fur ecosystem. Accurate time-resolved data of sloth movements in 3-dimensions (latitude, longitude, and altitude/elevation) is currently lacking, which limits a deeper understanding about how sloths move through the forest, their interactions with their environment and other animals, and their responses to habitat degradation or change (Santos et al., 2016; Pool et al., 2016; Brandão et al., 2019; Garcés-Restrepo et al., 2019b). Data on social and habitat connectivity are critical for understanding the sources and modes of symbiont transmission. Coupling movement (spatial geo-tracking) data with real-time local environmental sensing (or time-series data) of temperature, humidity, light, etc., and with periodic biodiversity surveys of sloth fur, would provide valuable insights into the degree of variation and environmental conditions that a sloth experiences vis-à-vis how the fur ecosystem is structured and changes.

5. Symbionts, Health, and Conservation

Sloth habitats are in danger of anthropogenic-induced destruction and climate change, with unknown consequences on sloths and their symbionts. Systematic research efforts are needed to determine by what means and by what mechanisms fur symbionts contribute to sloth health, and the impact of environmental or habitat changes. *B. pygmaeus* is critically endangered (Anderson and Handley, 2001; Hayssen, 2008), and the other five species are threatened by habitat loss and human encroachment. Additionally, sloths face many health challenges in captivity (de Stefani Munaó Diniz & Oliveira, 1999); misinformed practices at sloth rehabilitation facilities and zoos, such as bathing sloths routinely without a specific need, could be ridding them of beneficial fur symbionts and disrupting fur ecosystem balance in a manner that negatively impacts sloth well-being. Host-associated microbiota and symbionts are known to influence host evolution, development, and function (McFall-Ngai, 2014; Gilbert et al., 2015; Carthey et al., 2019), and are important to consider for conservation efforts to be efficacious (Redford et al., 2012; McFall-Ngai 2015). We currently lack answers to several fundamental questions related to sloth health and conservation: what is the role of the microbiome in buffering or dictating disease susceptibility of the host (Spor et al., 2011; Daskin & Alford, 2012; Huttenhower et al., 2012; Bissett et al., 2013; Rebollar et al. 2016; Antwis et al., 2017; Carthey et al., 2019)? How do host-associated microbes and arthropods interact with pathogens that might invade, and how do these dynamics influence infection or disease? Are sloth diseases polymicrobial in nature (Vayssier-Taussat et al., 2014) as we observe in diseases of other systems like corals (Sato et al. 2017; Meyer et al. 2017; Sweet et al., 2019)? How much of sloth disease and mortality (e.g., in captive animals) are related to microbiome dysbiosis (Levy et al., 2017; Hook & O'Malley 2017)? Studying the diversity of the sloth fur ecosystem and symbiont

community as a function of sloth health status will help us understand what members of the microbiome may be indicators of a healthy host.

Because symbiotic interactions are often context dependent, varying along a continuum and sometimes changing from mutualism to parasitism or vice versa (Bronstein, 1994; Kogel, Franken, & Hückelhoven, 2006; Leung & Poulin, 2008), it will be important to study how the different sloth-symbiont relationships differ depending on the study location and the particular abiotic and biotic context in which the interaction takes place. While classifying sloths' symbionts as mutualistic, commensal, or parasitic may seem like the best first step, going beyond simple classifications of sloth-symbiont relationships as merely 'positive' or 'negative' and determining the symbionts' potential for pathogenicity (Casadevall, 2017) will be helpful in understanding sloth health and will bring nuance to conservation efforts. It will also be helpful to investigate the resilience of the fur ecosystem to the sort of (fungal) infections that conservationists have observed sloths to suffer in the wild, and to understand to what degree these might be linked to anthropogenic disturbances that endanger the native habitat of sloths (Bissett et al., 2013). Microbiomes have been shown to be important in mammalian health and resilience (Fagundes et al., 2012; Kinross, Darzi, & Nicholson, 2011; McKenney et al., 2018; Round & Mazmanian, 2009). Research focusing on the health benefits of the sloth hair microbiome will be key in providing the best care for sloths in captivity and rehabilitation centers and may inform conservation initiatives to reintroduce captive animals to native habitats and to ensure the survival of sloths as unique ecosystems.

CONCLUSIONS

1) As a model mobile ecosystem, sloths are an intriguing example of a community of symbionts that could provide key insights into how and why these interactions form. To date, the relationships of these symbionts with sloths and with each other are poorly defined.
2) There is no clear empirical evidence showing the degree to which the algae growing in sloth fur is mutualistic or simply commensal, despite many hypotheses and proposals that attempt to describe the interaction.
3) Arthropods found in sloth hair can be commensals, mutualists, or parasites, but the interactions between these symbionts and the function of the arthropods in the sloth hair ecosystem are highly understudied.
4) The ecology of the fungi growing in sloth fur is perhaps the least studied realm of sloth symbiont research. While sloth fungi may have key benefits to humans, epibiotic sloth fungi may be parasitic, mutualistic, or commensal.
5) The prokaryotic component of the sloth fur ecosystem has not been characterized, although it has for the sloth gut microbiome. The similarities and connectedness of these two portions of the sloth holobiont are unknown.
6) It is unknown to what degree the sloth holobiont is a product of vertical transmission and coevolution with the sloth host vs. repeated re-assembly of similar taxa from the environment (ecological fitting) vs. a random chance sampling of environmental species pools. It is probable that the sloth holobiont assembles through a combination of these processes.

7) The sloth fur ecosystem is a poorly studied reservoir of potentially novel biodiversity. This includes novel taxa and genetic diversity that may in part code for unusual natural products of biotechnological relevance to agriculture and human health. These natural products may be specialized/secondary metabolites that support the chemical ecology of the sloth fur ecosystem and mediate interactions between microorganisms and arthropods.
8) Sloths present several opportunities as a model for symbiosis and ecological research, from holobiont/hologenome and niche theory, to network, metacommunity, and ecosystems ecology. Sloths are the only mammal with epibiotic growth on their hair that has been studied in any detail. The species-rich assemblage of microbes and arthropods on sloths provides a unique system for investigating how a multi-trophic network of interacting species assembles and coevolves.
9) Given sloths' unusually slow movements through a large horizontal and vertical space within a tropical forest ecosystem, sloths provide a unique window into an ecological and spatiotemporal regime not experienced by most other animals. Whether this combination of characteristics gives rise to the plethora of biodiversity associated with the sloth fur remains to be tested. Slow movements may facilitate colonization by microorganisms and arthropods and minimize subsequent dispersal. The sloth example highlights the importance of underlying ecosystem dynamics and process timescales on the biodiversity of the ecosystem.
10) Elucidating the basic ecology and fitness implications of the sloth microbiome may be fundamental to conservation initiatives, especially considering many sloth species face decline due to anthropogenic habitat loss. Importantly, there is the potential to discover

microbiome-associated predictive metrics of sloth health that may be of conservation value and to understand how the sloth fur ecosystem might endow sloths with resilience against environmentally induced stress.

CHAPTER II:

USING METAGENOMICS TO ELUCIDATE THE SLOTH HAIR ECOSYSTEM

INTRODUCTION

The diversity of microbial life on this planet is enormous, and metagenomic DNA sequencing has undeniably improved upon the culture-dependent efforts to catalogue this diversity (DeLong & Pace, 2001; Hirsch et al., 2010; Hugenholtz et al., 1998). Different DNA sequencing strategies yield differing results, however. For example, amplicon sequencing may give a snapshot of the diversity present in a sample but may not be sufficient for species-level resolution; whole-community shotgun metagenomic sequencing, on the other hand, could give a fuller representation of genetic and functional diversity, aid in the discovery of new species, and advance reference-free genome construction efforts (Eloe-Fadrosh et al., 2016; Quince et al., 2017; Shakya et al., 2013).

One area in which whole-community metagenomic sequencing has been particularly useful is in the field of microbiome studies (Baker & Dick, 2013). An animal's microbiome plays a key role in the health and fitness of its host (Barko et al., 2018; Lloyd-Price et al., 2016; Mueller & Sachs, 2015). Whole-community metagenomic studies further our understanding of microbial diversity and how it might influence the host by providing a way to measure and fully characterize the genomic repertoire of the microbiome (Baker & Dick, 2013; Cantarel et al., 2011). Sloths present a unique opportunity to further microbiome studies. While the gut microbiome of sloths has been surveyed and may play an important role in sloth health (Delsuc

et al., 2014; Dill-McFarland et al., 2016), the rich diversity of epibiotic symbionts on sloths is what makes them distinctive and it remains poorly understood.

Sloth fur may be a reservoir of unexplored microbial diversity, containing fungi, algae, and undoubtedly bacteria, although no bacterial survey has been performed (Higginbotham et al., 2014; Kaup et al., 2020; Suutari et al., 2010), and data from whole-community metagenomic sequencing can be used to study all of these epibionts at once. Two closely-related species of sloths, *Bradypus variegatus* and *Choloepus hoffmanni*, living in sympatry may differ in community composition at the level of the fur microbiome. The two species of sloths' convergent evolution (Delsuc et al., 201; Presslee et al., 2019) make their similarities and differences in hair microbiomes particularly interesting to study. Fountain et al. (2017) have proposed that one species of green algae has coevolved with its host sloth, although this conjecture lacks concrete evidence; their work is the only attempt to investigate the coevolution of sloth species and their fur microbiome.

The algal diversity growing in the fur of sloths has almost exclusively been identified using morphology and amplicon sequencing techniques, which have led to the description of fourteen species of green algae across all six species of sloths (Suutari et al., 2010; Thompson, 1972). One particular green alga, *Trichophilus welckeri*, is described as the algal species responsible for the green coloration on most three-fingered sloths' pelage (Weber-van Bosse, 1887), and, in fact, is the only green alga identified to date on three of the four species of three-fingered sloths (Aiello, 1985; Pauli et al., 2014; Suutari et al., 2010). Other species of green algae have been identified, but it is unknown from which species of sloth these specimens originated (Thompson, 1972; Suutari et al., 2010). The brown-throated three-fingered sloth, *B. variegatus*, has been hypothesized to consume the *T. welckeri* growing on their fur as a source of

nutrition due to their limited gut size and a diet of leaves with little nutritional value (Pauli et al., 2014).

Similarly, the roles and diversity of fungi and bacteria in the sloth hair microbiome are poorly known. Amplicon sequencing with 18S, ITS, and LSU rRNA genes of sloth hair fungi have yielded a baseline for understanding the fungal diversity present (Higginbotham et al., 2014; Suutari et al., 2010), but often these fungi are not identifiable to the species level and the differences in fungal diversity between sloth species has not been characterized. Fungi from *B. variegatus* have been hypothesized to be a potential source for new compounds for drug development (Higginbotham et al., 2014), but it is unclear whether these fungi benefit or harm the sloth, or the other symbionts in the sloth hair microbiome. Besides one species of cyanobacteria (Wujek & Lincoln, 1988), the bacteria in sloth hair have not been studied.

To clarify the microbial diversity of the sloth hair microbiome for both *B. variegatus* and Hoffmann's two-fingered sloth, *C. hoffmanni*, I used whole-community shotgun metagenomic sequencing of sloth hair collected in Manuel Antonio, Costa Rica. I hypothesized that the hair microbiomes of two- and three-fingered sloths are different, and that there are differences that depend on the location on the sloth that was sampled. My results suggest that the diversity of the sloth hair microbiome and its role in the sloth hair ecosystem may have previously been oversimplified owing to my discovery of a vast array of microorganisms represented in the metagenome of sloth hairs.

METHODS

Sample Collection

Sloth hair samples were collected with approval from the University of Mississippi Institutional Animal Care and Use Committee (Protocol #18-005). Eleven wild adult sloths of two species, *B. variegatus* (n=6) and *C. hoffmanni* (n=5), were caught during the dry season (January-May) with help from volunteers at The Sloth Institute in Manuel Antonio, Costa Rica (Table 1), under Resolution #ACOPAC-INV-001-17 from the Ministerio de Ambiente, Energia y Telecomunicaciones, Sistema Nacional de Áreas de Conservación, Costa Rica. The study location was Tulemar Resort, a 13.35 hectare property on which The Sloth Institute is located. The property is predominantly maritime rainforest habitat with many native trees. Hair samples were imported to the United States under USDA Permit # P526P-15-03183. Sloths were caught when they descended to the base of a tree to defecate, or were retrieved from the canopy of trees using a 6 m ladder. Sloths were placed in a soft-sided carrier as they were lowered from the tree. Using scissors sterilized with 70% isopropanol, a 1 cm^2 patch of hair was clipped from six wild *B. variegatus* and five *C. hoffmanni* from the greenest areas of the sloth, the shoulder and back of the head. Hair of each of the 22 samples (11 sloths with shoulder and head samples for each) was placed aseptically in sterile Whirl-Pak bags (Nasco, WI) until further processing (see below).

Table 1. Collection details for sloth hair samples used in this study. Hair samples were collected from both head and shoulder locations for each sloth.

Sample ID	Sloth Species	Sex	Collection Location	Observations/Notes
CH01	*Choloepus hoffmanni*	F	N 09°24.490´ W 084°09.701´	Young adult, no visible algae
CH02	*Choloepus hoffmanni*	F	N 09°24.458´ W 084°09.791´	Adult, slightly green on shoulders
CHO3	*Choloepus hoffmanni*	F	N 09°24.434´ W 084°09.598´	Adult, pregnant, no visible green
CH04	*Choloepus hoffmanni*	M	N 09°24.448´ W 084°09.659´	Adult, no visible green
CH05	*Choloepus hoffmanni*	F	N 09°24.567´ W 084°09.756´	Adult, no visible green, hand-raised and released
BV01	*Bradypus variegatus*	M	N 09°24.544´ W 084°09.624´	Adult, visible green on head & shoulders
BV02	*Bradypus variegatus*	M	N 09°24.500´ W 084°09.627´	Adult, visible green on head & shoulders
BV03	*Bradypus variegatus*	M	N 09°24.512´ W 084°09.714´	Adult, visible green on head & shoulders
BV04	*Bradypus variegatus*	F	N 09°24.459´ W 084°09.716´	Young adult, no visible green, rescued from the sloth-selfie trade
BV05	*Bradypus variegatus*	F	N 09°24.487´ W 084°09.797´	Adult, no visible green
BV06	*Bradypus variegatus*	M	N 09°24.468´ W 084°09.508´	Adult, visible green on head and shoulders, fungal skin infection

DNA Extraction and Quantification

Hair was placed in 1.5 mL of DNA preservation buffer (ammonium sulfate [3.8 M], EDTA disodium salt dihydrate [0.25 mM], sodium citrate dihydrate, and citric acid, anhydrous [final citrate buffer = 50 mM] (modified based on Camacho-Sanchez et al. 2013) within 24 hours of collection and stored at -18°C until transport (<2 months) and at -30°C until DNA could be extracted (<10 months). Prior to performing DNA extractions, preservation buffer was removed by pipetting and hair was gently rinsed by suspension in 1.5 mL of PBS (<1 min). A Macherey-Nagel NucleoSpin® Soil kit was used to extract DNA from each lock of hair. The standard

protocol for the kit was followed, with the exception of using three rounds of homogenization for 30 s at 3,000 oscillations per minute on a Mini-Beadbeater-24 (Biospec Products, Inc., OK) instead of 5 min on a vortexer. Isolated DNA was quantified using a QuantiFluor dsDNA Kit on a Quantus Fluorometer (Promega, Inc., WI) and stored at -30°C.

Library Preparation and DNA Sequencing

The quantity of DNA in each sample was standardized to 100 ng and prepared for Illumina sequencing using a NEBNext Ultra II FS DNA Library Prep Kit (New England Biolabs, Inc., MA). I used a 15 min fragmentation time, a 25 µL (step 1) / 10 µL (step 2) bead ratio for size selection, and five cycles of PCR. Library quality was assessed on a 2100 BioAnalyzer (Agilent Technologies, Inc., CA) using a High Sensitivity DNA Kit (5067-4626) to ensure proper fragmentation of DNA and the absence of adapters and primer dimers, and to quantify DNA. Library quantifications were also cross-validated via qPCR using a NEBNext Library Quant Kit (E7630S). Libraries were pooled, "cleaned" a final time using NEBNext Sample Purification Beads (E7775S) following manufacturer instructions, and sequenced: first using Illumina MiSeq for quality control, and subsequently using one lane of a Illumina NovaSeq 6000 S4 flow cell with 2x150 bp paired-end reads (GENEWIZ, NJ).

Bioinformatic Sequence Processing

Raw sequences were trimmed and cleaned using Trimmomatic (version 0.39) and Sickle (version 1.33) (Bolger et al., 2014; Joshi & Fash, 2011). Cleaned reads were assigned taxonomic classification using Kaiju (version 1.7.2) and the NCBI non-redundant eukaryotic database

(nr_euk, downloaded December, 2019), which included bacteria, archaea, viruses, fungi, and eukaryotes (Menzel et al., 2016).

Statistical Analyses

R (version 3.5.3) was used to convert Kaiju outputs (kaiju2table.txt) into count tables, package *ggplot2* for creating bar charts and NMDS plots, and *vegan* for performing PERMANOVAs (see Appendix for code). All unclassified species were removed and reads were normalized (reads were converted to proportions based on the total number of sequenced reads for each sample) before creating the NMDS plot, performing the PERMANOVAs, and calculating diversity index metrics. A "whole plot" (or between-subjects) PERMANOVA (with 999 permutations) was run on centroids essentially averaging head and shoulder samples to determine overall differences between sloth species. A repeated measurement permutation MANOVA ("split plot"; S. Brewer, personal communication) was run to account for pseudo-replicated samples from each sloth (from head and shoulder) and to test for a correlated interaction between location sampled on the sloth (head or shoulder) and the type of sloth (two-fingered or three-fingered). Inverse Simpson's and Shannon's diversity indices (Shannon, 1948; Simpson, 1949) were calculated using *vegan*, given that they are the most widely accepted diversity indices (Chernov et al., 2015; Gorelick, 2006). Welch's t-test (Welch, 1947) was used to determine statistical significance of differences in the number of reads between two- and three-fingered sloths, the amount of chlorophyte algae on two- versus three-fingered sloths based on normalized reads, and diversity index metrics.

RESULTS

Hair samples from two body locations (head and shoulder) on five *B. variegatus* and six *C. hoffmanni* were analyzed. Each hair metagenome sample was sequenced with a random and blinded block design to an average depth of 61.7 million paired-end reads (2x150 bp) or 18.5 Gb of sequence/sample. The number of reads for two- versus three-fingered sloths was not significantly different ($p = 0.219$). Unclassified organisms or ambiguous assignments accounted for 58% of sequence reads in *C. hoffmanni* and 60% in *B. variegatus*. Bacteria were the most dominant classified microbial component of the hair microbiome, composing on average 40% of the total reads in the sloth hair microbiome across both species of sloths, while Eukaryota composed 1%, Archaea 0.08%, and viruses 0.02%.

Bacteria accounted for 38-41% of all sample reads in two- and three-fingered sloths (Figure 1A). Bacterial species found on sloths were mainly from the phyla Proteobacteria (25-34%), Actinobacteria (27-30%), Bacteroidetes (11-18%), Acidobacteria (10-11%), and Firmicutes (6-8%; Figure 1A). The proportions of Bacteroidetes (18%) and Firmicutes (8%) were higher in *C. hoffmanni* (*B. variegatus* have 11% and 7%, respectively), while *B. variegatus* have slightly higher proportions of Proteobacteria (34% compared to 26%) and Actinobacteria (30% compared to 27%). Archaea accounted for 0.080-0.088% of all sample reads (Figure 1B). Euryarchaeota was the most prominent archaeal phyla (73-77%), followed by Thaumarchaeota (4-9%) and Crenarchaeota (5%); *C. hoffmanni* had fewer Euryarchaeota reads (73%), but more Thaumarchaeota (9%) than their three-fingered counterparts (77% and 4%, respectively; Figure 1B). Three-fingered sloths had slightly more fungi in their fur microbiome than two-fingered sloths (0.827% vs. 0.74%, Figure 1C). Ascomycota (71-79%) and Basidiomycota (17-21%) were the dominant fungal phyla, with *C. hoffmanni* having a slightly higher proportion of Ascomycota

(79%) and *B. variegatus* having a slightly higher proportion of Basidiomycota (21%; Figure 1C). The main photosynthetic microbial phyla ("algae") found on both species of sloths were Chlorophyta (41-43%), Rhodophyta (25-33%), and Euglenozoa (~10%), although there were many other phyla that were broadly grouped as "algae," comprising photosynthetic protist clades that were found on both species of sloths (Figure 1D). *B. variegatus* had over twice as much "algae" as *C. hoffmanni* in their fur microbiome (0.44% vs 0.18%, Figure 1D). *B. variegatus* had a significantly higher number of reads of chlorophytes (relative to total sample reads: 0.18±0.02%) present in their hair microbiome than *C. hoffmanni* (0.074±0.008%; Figure 1E; $p < 0.0001$). Within the class Chlorophyta, *C. hoffmanni* had a higher proportion of Chlorophyceae (50% compared to 41%), but a lower proportion of Trebouxiophyceae (25% compared to 32%) and Ulvophyceae (15% compared to 18%) when compared to *B. variegatus* (Figure 1). *B. variegatus* had three times as many rhodophyte reads as *C. hoffmanni* (0.15% vs. 0.044%, Figure 1F). The top three classes of Rhodophyta were Bangiophyceae (more in *C. hoffmanni*, 52% compared to 35%), Florideophyceae (more in *B. variegatus*, 35% compared to 26%), and Stylonematophyceae (more in *B. variegatus*, 25% compared to 18%, Figure 1F).

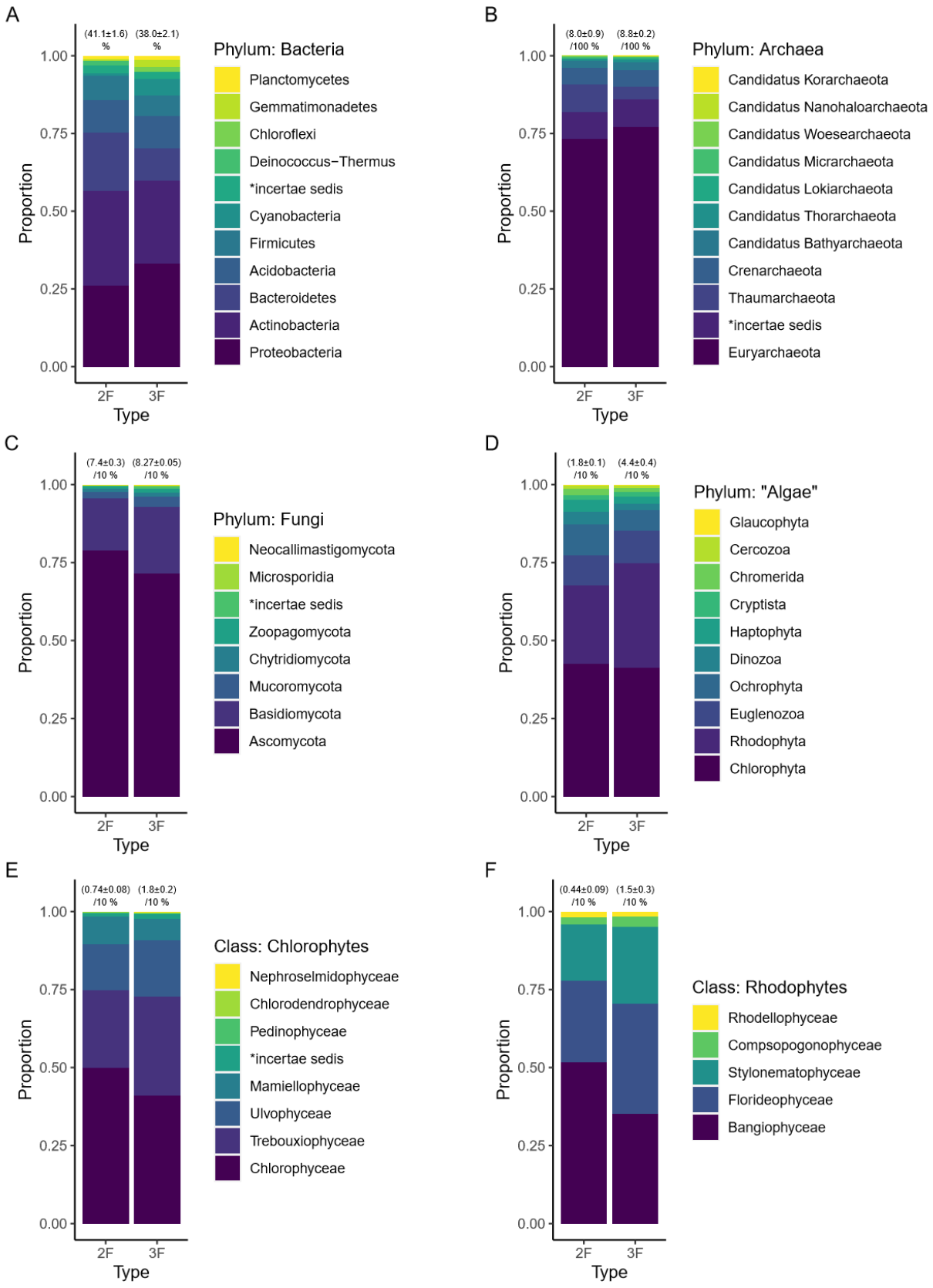

A
(41.1±1.6) %
(38.0±2.1) %
Proportion
1.00
0.75
0.50
0.25
0.00
2F
3F
Type
Phylum: Bacteria
Planctomycetes
Gemmatimonadetes
Chloroflexi
Deinococcus−Thermus
*incertae sedis
Cyanobacteria
Firmicutes
Acidobacteria
Bacteroidetes
Actinobacteria
Proteobacteria
B
(8.0±0.9) /100 %
(8.8±0.2) /100 %
Phylum: Archaea
Candidatus Korarchaeota
Candidatus Nanohaloarchaeota
Candidatus Woesearchaeota
Candidatus Micrarchaeota
Candidatus Lokiarchaeota
Candidatus Thorarchaeota
Candidatus Bathyarchaeota
Crenarchaeota
Thaumarchaeota
*incertae sedis
Euryarchaeota
C
(7.4±0.3) /10 %
(8.27±0.05) /10 %
Phylum: Fungi
Neocallimastigomycota
Microsporidia
*incertae sedis
Zoopagomycota
Chytridiomycota
Mucoromycota
Basidiomycota
Ascomycota
D
(1.8±0.1) /10 %
(4.4±0.4) /10 %
Phylum: "Algae"
Glaucophyta
Cercozoa
Chromerida
Cryptista
Haptophyta
Dinozoa
Ochrophyta
Euglenozoa
Rhodophyta
Chlorophyta
E
(0.74±0.08) /10 %
(1.8±0.2) /10 %
Class: Chlorophytes
Nephroselmidophyceae
Chlorodendrophyceae
Pedinophyceae
*incertae sedis
Mamiellophyceae
Ulvophyceae
Trebouxiophyceae
Chlorophyceae
F
(0.44±0.09) /10 %
(1.5±0.3) /10 %
Class: Rhodophytes
Rhodellophyceae
Compsopogonophyceae
Stylonematophyceae
Florideophyceae
Bangiophyceae

Figure 1. Phylogenetic composition of the microbial community on sloth hair between all *C. hoffmanni* (2F, which stands for two-fingered) and *B. variegatus* (3F, which stands for three-fingered) samples of A) bacterial phyla; B) archaeal phyla; C) fungal phyla; D) all "algal" phyla, which I interpreted broadly to include any phylum (excluding cyanobacteria) that contained photosynthetic representatives; E) chlorophyte classes; and F) rhodophyte classes. Only the top ten most prevalent bacterial, archaeal, and "algal" phyla are shown. *Incertae sedis* indicates an assortment of taxa with an uncertain or unresolved phylogenetic placement. Percentage (± standard deviation) values at the top of stacked bar charts denote the average proportion of reads assigned to that taxon relative to *all* reads per sample collected for that sloth type (N=10 for 2F and N=12 for 3F (head or shoulder samples)).

Table 2. Tally of previously identified and currently identified (from this study) genera of rhodophytes, chlorophytes, fungi, cyanobacteria, and other bacteria associated with the sloth fur microbiome. Numbers represent total genera across all sampled sloths, excluding singletons. Previously identified rhodophyte data is from Wujek & Timpano (1986). Previously identified green algae and cyanobacteria data are from Suutari et al. (2010) (as clarified through personal correspondence with M. Suutari and J. Blomster) and Wujek & Lincoln (1988). Eleven genera of chlorophytes and cyanobacteria were previously found on sloths, but are of an unidentified origin and thus are not listed below (see Table 3 in Chapter I instead; Thompson, 1972; Suutari et al., 2010). Fungal data is from Higginbotham et al. (2014). Fungi were also identified by Suutari et al. (2010), but it is ambiguous from which sloth species they were derived.

	Hoffmann's two-fingered sloth (*Choloepus hoffmanni*)		**Brown-throated three-fingered sloth (*Bradypus variegatus*)**	
	Previously identified	**Currently identified**	**Previously identified**	**Currently identified**
Rhodophytes	1	187	1	255
Chlorophytes	3	222	2	251
Fungi	Not clear	633	16	808
Cyanobacteria	1	95	1	113
Other Bacteria	0	2363	0	2369

Our sequencing results revealed at least 100-fold more genera of red algae, green algae, fungi, cyanobacteria, and other bacteria on sloth hair than previously identified through amplicon-based studies (Table 2). The numbers of genera in each of these groups were roughly similar between two- and three-fingered sloths. The number of species in each of these groups and the top three taxa hits to the non-redundant NCBI database (nr_euk) as assigned by Kaiju are also similar across species (Table 3).

Clustering using Bray-Curtis dissimilarities in a non-metric multidimensional scaling (NMDS) ordination showed distinct grouping of hair microbiome communities of two- vs. three-fingered sloths (Figure 3). The whole plot, or between-subjects, PERMANOVA showed significant community differences between two- and three-fingered sloths (PERMANOVA r^2 = 0.343, p = 0.007). A repeated measurement permutation MANOVA ("split-plot") revealed no significant difference in community composition between the different locations (head or shoulder) on an individual sloth basis (PERMANOVA r^2 = 0.011, p = 0.616). There was also no significant interaction between the location sampled on the sloth and the species of sloth (PERMANOVA r^2 = 0.017, p = 0.279). *C. hoffmanni* had a more diverse microbiome when using the Inverse Simpson's index (p = 0.004). In contrast, Shannon's diversity index showed no significant difference in diversity between the two sloths' fur microbiomes (p = 0.147) (Table 4).

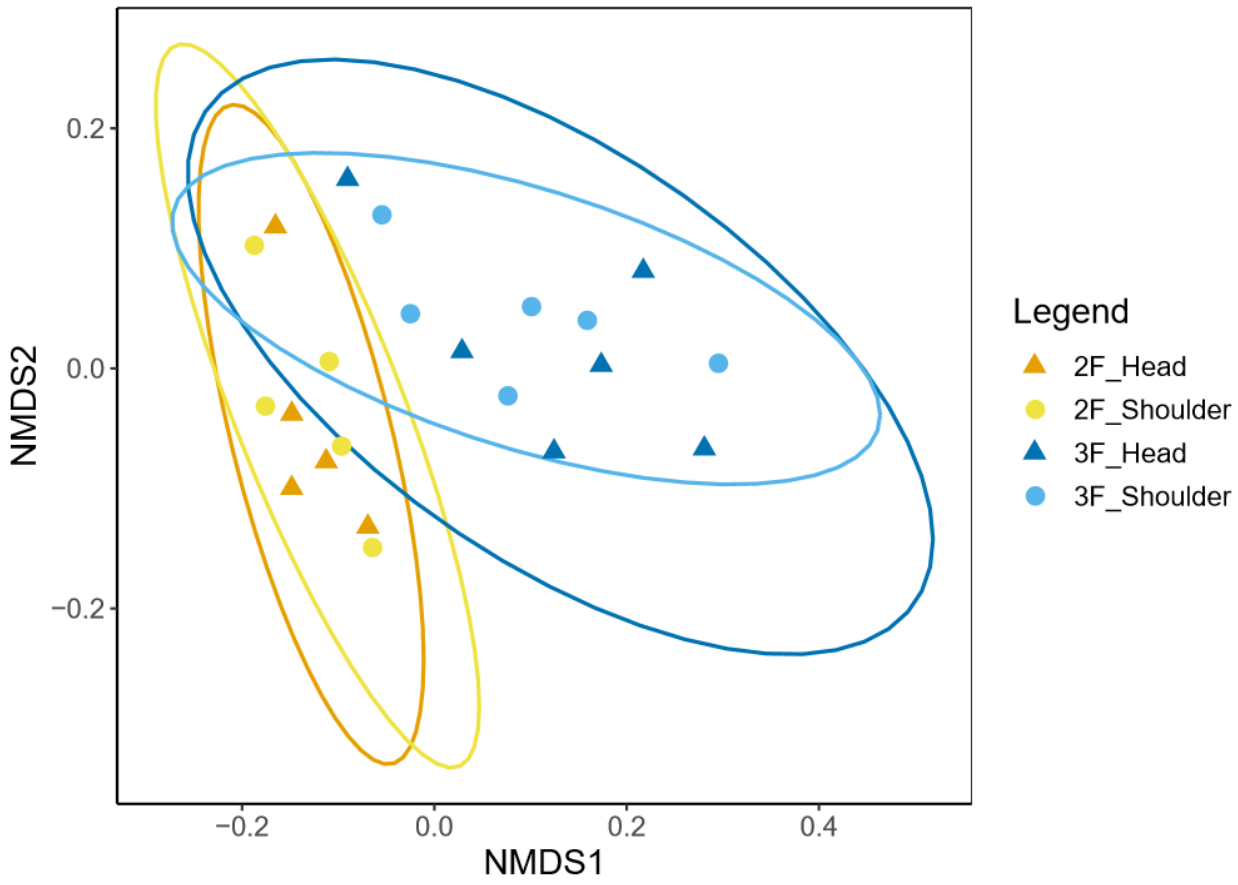


Figure 2. Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities at the species level comparing two- and three-fingered sloths, as well as the location sampled on the sloth (head vs. shoulder). Each point represents a sample. 2F denotes the two-fingered sloth (*C. hoffmanni*) and 3F denotes the three-fingered sloth (*B. variegatus*). Ellipses outline a 95% confidence interval around data centroids.

Table 3. Number of species of rhodophytes, chlorophytes, fungi, cyanobacteria, and other bacteria associated with sloth fur are shown (excluding singletons). The top three taxa/matches to the non-redundant NCBI (nr_euk) database assigned by Kaiju are listed in descending order.

	Hoffmann's two-fingered sloth (*Choloepus hoffmanni*)		**Brown-throated three-fingered sloth (*Bradypus variegatus*)**	
	# of species	**Most common species (in order)**	**# of species**	**Most common species (in order)**
Rhodophytes	306	*Porphyra umbilicalis* *Bangiopsis subsimplex* *Chondrus crispus*	459	*Bangiopsis subsimplex* *Porphyra umbilicalis* *Chondrus crispus*
Chlorophytes	434	*Chlamydomonas reinhardtii* *Raphidocelis subcapitata* *Gonium pectorale*	578	*Coccomyxa subellipsoidea* *Gonium pectorale* *Chlamydomonas eustigma*
Fungi	1431	*Saccharomycodes ludwigii* *Cyphellophora europaea* *Phialophora attae*	1838	*Saccharomycodes ludwigii* *Hortaea werneckii* *Verruconis gallopava*
Cyanobacteria	440	cyanobacterium TDX16 *Hassallia byssoidea* oscillatoriacean cyanobacterium	572	*Aliterella atlantica* *Synechocystis* sp. PCC 7509 *Chroococcidiopsis cubana*
Other Bacteria	24994	Acidobacteria bacterium *Enterococcus faecium* Chitinophagaceae bacterium	25359	Acidobacteria bacterium *Enterococcus faecium* *Gemmatirosa kalamazoonesis*

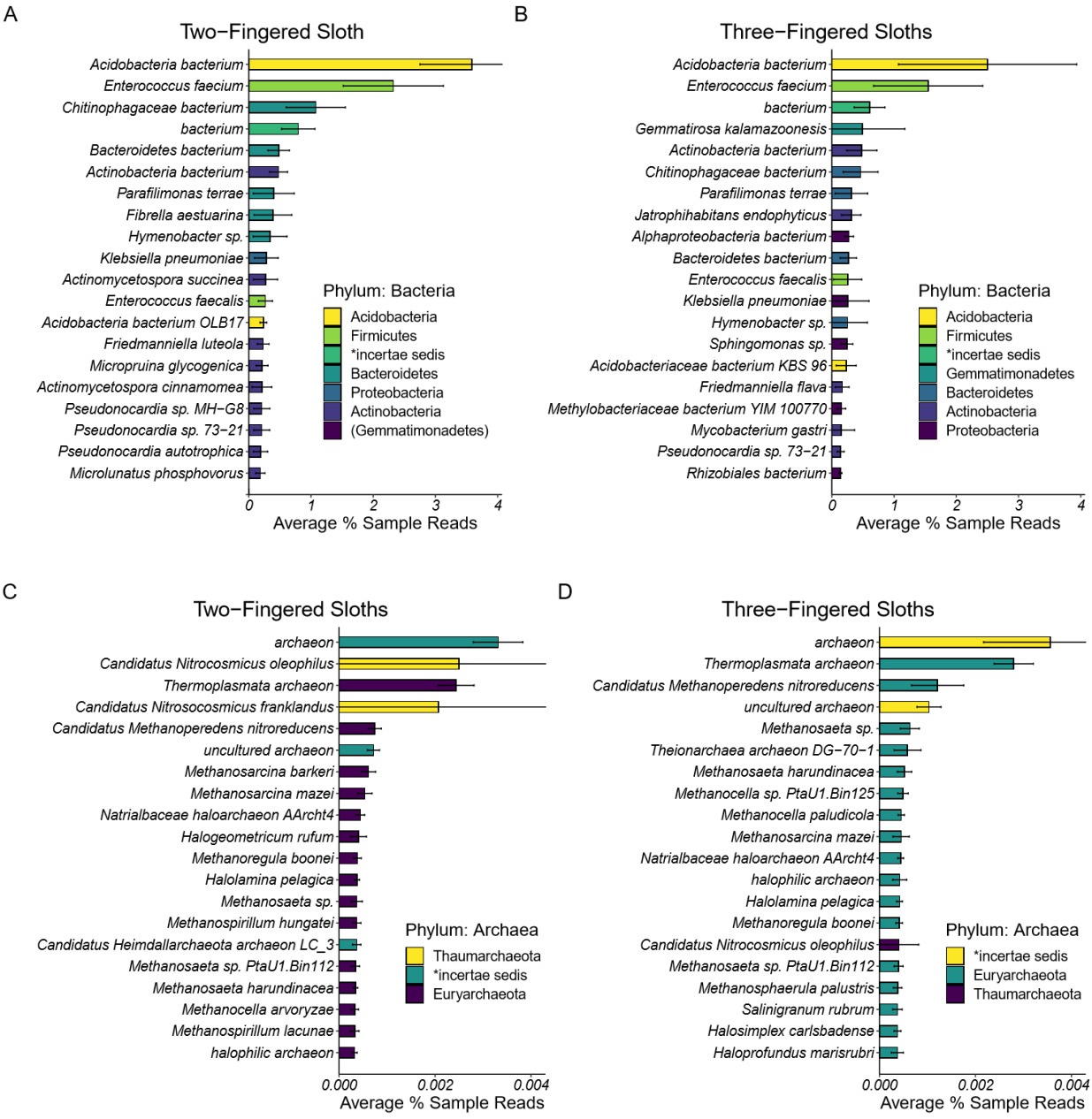
Table 4. Calculated diversity indices for sloth hair microbiome communities at the species level for *C. hoffmanni* and *B. variegatus*. Statistical significance (as determined by t-test with p-value = 0.004) is indicated by an asterisk.

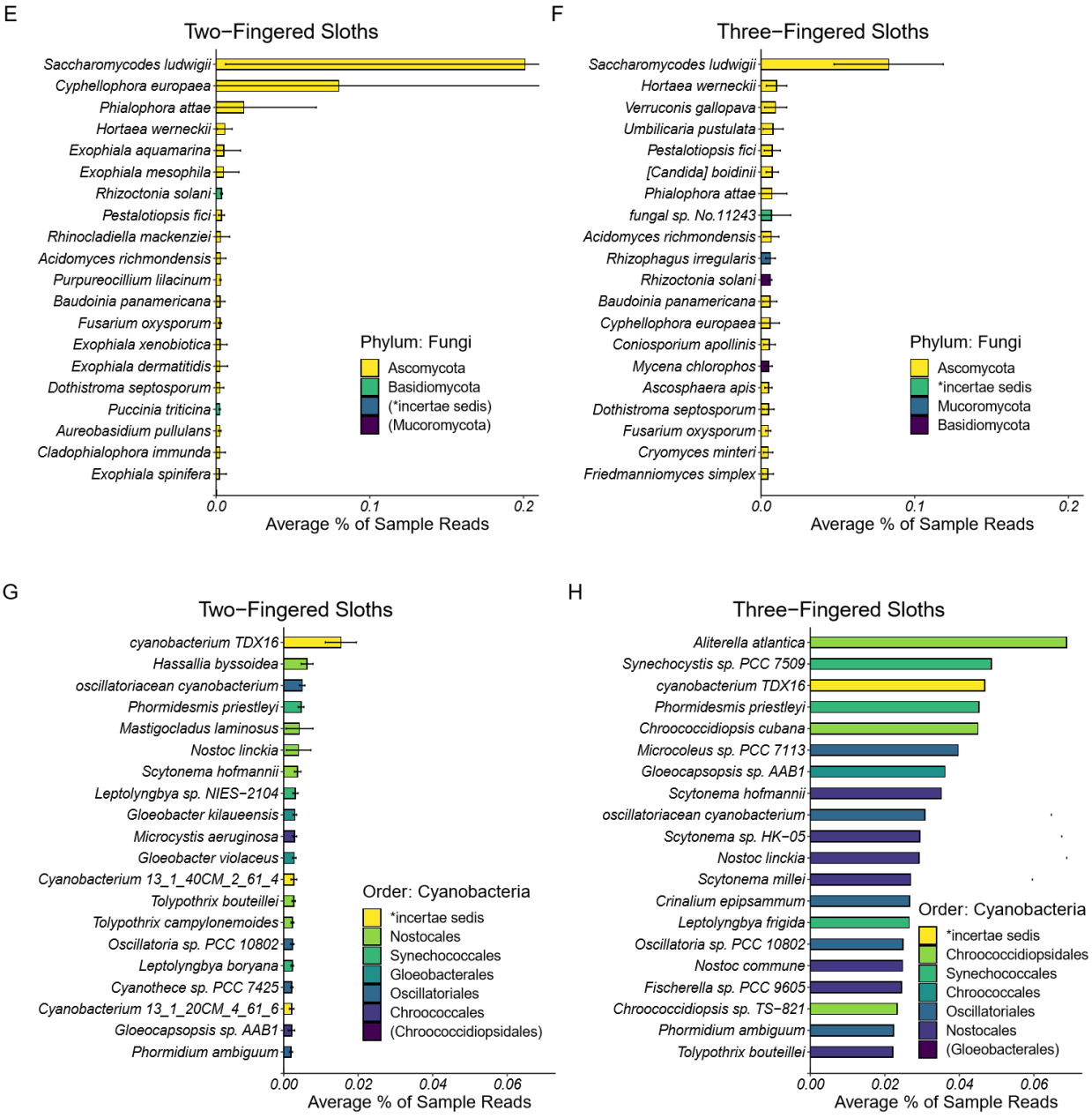
	Inverse Simpson's Index	**Shannon's Index**
C. hoffmanni	7.3 ± 0.4*	5.11 ± 0.08
B. variegatus	6.7 ± 0.5*	5.2 ± 0.2

C. hoffmanni and *B. variegatus* hair microbiomes shared two of the most prevalent bacterial species (as designated by Kaiju matches to the nr_euk database): an unknown member of the Acidobacteria and *Enterococcus faecium* (Table 3; Figure 3A, B). The most prevalent species of Archaea for both sloth species was an unidentified archaeon; the second most

prevalent species for *C. hoffmanni* was *Candidatus* Nitrocosmicus oleophilus and for *B. variegatus*, an unidentified Thermoplasmata archaeon (Figure 3C, D). *Saccharomycodes ludwigii* was the most prevalent fungus on both sloth species, followed by *Cyphellophora europaea* on *C. hoffmanni* and *Hortaea werneckii* on *B. variegatus* (Table 3; Figure 3E, F). Cyanobacterial species differed, with an unclassifical cyanobacterium and *Hassalia byssoidea* being the most prevalent species on *H. hoffmanni*, with *Aliterella atlantica* and *Synechocystis* sp PCC 7509 being the most prevalent species on *B. variegatus* (Table 3; Figure 3G, H). For chlorophyte algal species in order of decreasing prevalence: *Chlamydomonas reinhardtii, Raphidocelis subcapitata*, and *Gonium pectorale* were the most prevalent on *C. hoffmanni*, while *Coccomyxa subellipsoidea*, *Gonium pectorale*, and *Chlamydomonas eustigma* were the most prevalent on *B. variegatus* (Table 3; Figure 3I, J). Although the order is not identical, the top three species of rhodophytes were *Porphyra umbilicalis*, *Bangiopsis subsimplex*, and *Chondrus crispus* for both species of sloths (Table 3; Figure 3K, L).

There were also many microbial symbionts other than bacteria, archaea, fungi, green algae, and red algae found in sloth fur. Microbes from 26 other phyla were identified from fur of both *B. variegatus* and *C. hoffmanni*; nine of these phyla contain photosynthetic microorganisms (Table 5). The vast majority of these symbionts have not previously been identified to be associated with sloths (see Table 4 in Chapter I).


A
Two−Fingered Sloth
Acidobacteria bacterium
Enterococcus faecium
Chitinophagaceae bacterium
bacterium
Bacteroidetes bacterium
Actinobacteria bacterium
Parafilimonas terrae
Fibrella aestuarina
Hymenobacter sp.
Klebsiella pneumoniae
Actinomycetospora succinea
Enterococcus faecalis
Acidobacteria bacterium OLB17
Friedmanniella luteola
Micropruina glycogenica
Actinomycetospora cinnamomea
Pseudonocardia sp. MH−G8
Pseudonocardia sp. 73−21
Pseudonocardia autotrophica
Microlunatus phosphovorus
Phylum: Bacteria
Acidobacteria
Firmicutes
*incertae sedis
Bacteroidetes
Proteobacteria
Actinobacteria
(Gemmatimonadetes)
0
1
2
3
4
Average % Sample Reads
B
Three−Fingered Sloths
Acidobacteria bacterium
Enterococcus faecium
bacterium
Gemmatirosa kalamazoonesis
Actinobacteria bacterium
Chitinophagaceae bacterium
Parafilimonas terrae
Jatrophihabitans endophyticus
Alphaproteobacteria bacterium
Bacteroidetes bacterium
Enterococcus faecalis
Klebsiella pneumoniae
Hymenobacter sp.
Sphingomonas sp.
Acidobacteriaceae bacterium KBS 96
Friedmanniella flava
Methylobacteriaceae bacterium YIM 100770
Mycobacterium gastri
Pseudonocardia sp. 73−21
Rhizobiales bacterium
Phylum: Bacteria
Acidobacteria
Firmicutes
*incertae sedis
Gemmatimonadetes
Bacteroidetes
Actinobacteria
Proteobacteria
0
1
2
3
4
Average % Sample Reads
C
Two−Fingered Sloths
archaeon
Candidatus Nitrocosmicus oleophilus
Thermoplasmata archaeon
Candidatus Nitrososocosmicus franklandus
Candidatus Methanoperedens nitroreducens
uncultured archaeon
Methanosarcina barkeri
Methanosarcina mazei
Natrialbaceae haloarchaeon AArcht4
Halogeometricum rufum
Methanoregula boonei
Halolamina pelagica
Methanosaeta sp.
Methanospirillum hungatei
Candidatus Heimdallarchaeota archaeon LC_3
Methanosaeta sp. PtaU1.Bin112
Methanosaeta harundinacea
Methanocella arvoryzae
Methanospirillum lacunae
halophilic archaeon
Phylum: Archaea
Thaumarchaeota
*incertae sedis
Euryarchaeota
0.000
0.002
0.004
Average % Sample Reads
D
Three−Fingered Sloths
archaeon
Thermoplasmata archaeon
Candidatus Methanoperedens nitroreducens
uncultured archaeon
Methanosaeta sp.
Theionarchaea archaeon DG−70−1
Methanosaeta harundinacea
Methanocella sp. PtaU1.Bin125
Methanocella paludicola
Methanosarcina mazei
Natrialbaceae haloarchaeon AArcht4
halophilic archaeon
Halolamina pelagica
Methanoregula boonei
Candidatus Nitrocosmicus oleophilus
Methanosaeta sp. PtaU1.Bin112
Methanosphaerula palustris
Salinigranum rubrum
Halosimplex carlsbadense
Haloprofundus marisrubri
Phylum: Archaea
*incertae sedis
Euryarchaeota
Thaumarchaeota
0.000
0.002
0.004
Average % Sample Reads


E
Two−Fingered Sloths
Saccharomycodes ludwigii
Cyphellophora europaea
Phialophora attae
Hortaea werneckii
Exophiala aquamarina
Exophiala mesophila
Rhizoctonia solani
Pestalotiopsis fici
Rhinocladiella mackenziei
Acidomyces richmondensis
Purpureocillium lilacinum
Baudoinia panamericana
Fusarium oxysporum
Exophiala xenobiotica
Exophiala dermatitidis
Dothistroma septosporum
Puccinia triticina
Aureobasidium pullulans
Cladophialophora immunda
Exophiala spinifera
Phylum: Fungi
Ascomycota
Basidiomycota
(*incertae sedis)
(Mucoromycota)
0.0
0.1
0.2
Average % of Sample Reads
F
Three−Fingered Sloths
Saccharomycodes ludwigii
Hortaea werneckii
Verruconis gallopava
Umbilicaria pustulata
Pestalotiopsis fici
[Candida] boidinii
Phialophora attae
fungal sp. No.11243
Acidomyces richmondensis
Rhizophagus irregularis
Rhizoctonia solani
Baudoinia panamericana
Cyphellophora europaea
Coniosporium apollinis
Mycena chlorophos
Ascosphaera apis
Dothistroma septosporum
Fusarium oxysporum
Cryomyces minteri
Friedmanniomyces simplex
Phylum: Fungi
Ascomycota
*incertae sedis
Mucoromycota
Basidiomycota
0.0
0.1
0.2
Average % of Sample Reads
G
Two−Fingered Sloths
cyanobacterium TDX16
Hassallia byssoidea
oscillatoriacean cyanobacterium
Phormidesmis priestleyi
Mastigocladus laminosus
Nostoc linckia
Scytonema hofmannii
Leptolyngbya sp. NIES−2104
Gloeobacter kilaueensis
Microcystis aeruginosa
Gloeobacter violaceus
Cyanobacterium 13_1_40CM_2_61_4
Tolypothrix bouteillei
Tolypothrix campylonemoides
Oscillatoria sp. PCC 10802
Leptolyngbya boryana
Cyanothece sp. PCC 7425
Cyanobacterium 13_1_20CM_4_61_6
Gloeocapsopsis sp. AAB1
Phormidium ambiguum
Order: Cyanobacteria
*incertae sedis
Nostocales
Synechococcales
Gloeobacterales
Oscillatoriales
Chroococcales
(Chroococcidiopsidales)
0.00
0.02
0.04
0.06
Average % of Sample Reads
H
Three−Fingered Sloths
Aliterella atlantica
Synechocystis sp. PCC 7509
cyanobacterium TDX16
Phormidesmis priestleyi
Chroococcidiopsis cubana
Microcoleus sp. PCC 7113
Gloeocapsopsis sp. AAB1
Scytonema hofmannii
oscillatoriacean cyanobacterium
Scytonema sp. HK−05
Nostoc linckia
Scytonema millei
Crinalium epipsammum
Leptolyngbya frigida
Oscillatoria sp. PCC 10802
Nostoc commune
Fischerella sp. PCC 9605
Chroococcidiopsis sp. TS−821
Phormidium ambiguum
Tolypothrix bouteillei
Order: Cyanobacteria
*incertae sedis
Chroococcidiopsidales
Synechococcales
Chroococcales
Oscillatoriales
Nostocales
(Gloeobacterales)
0.00
0.02
0.04
0.06
Average % of Sample Reads

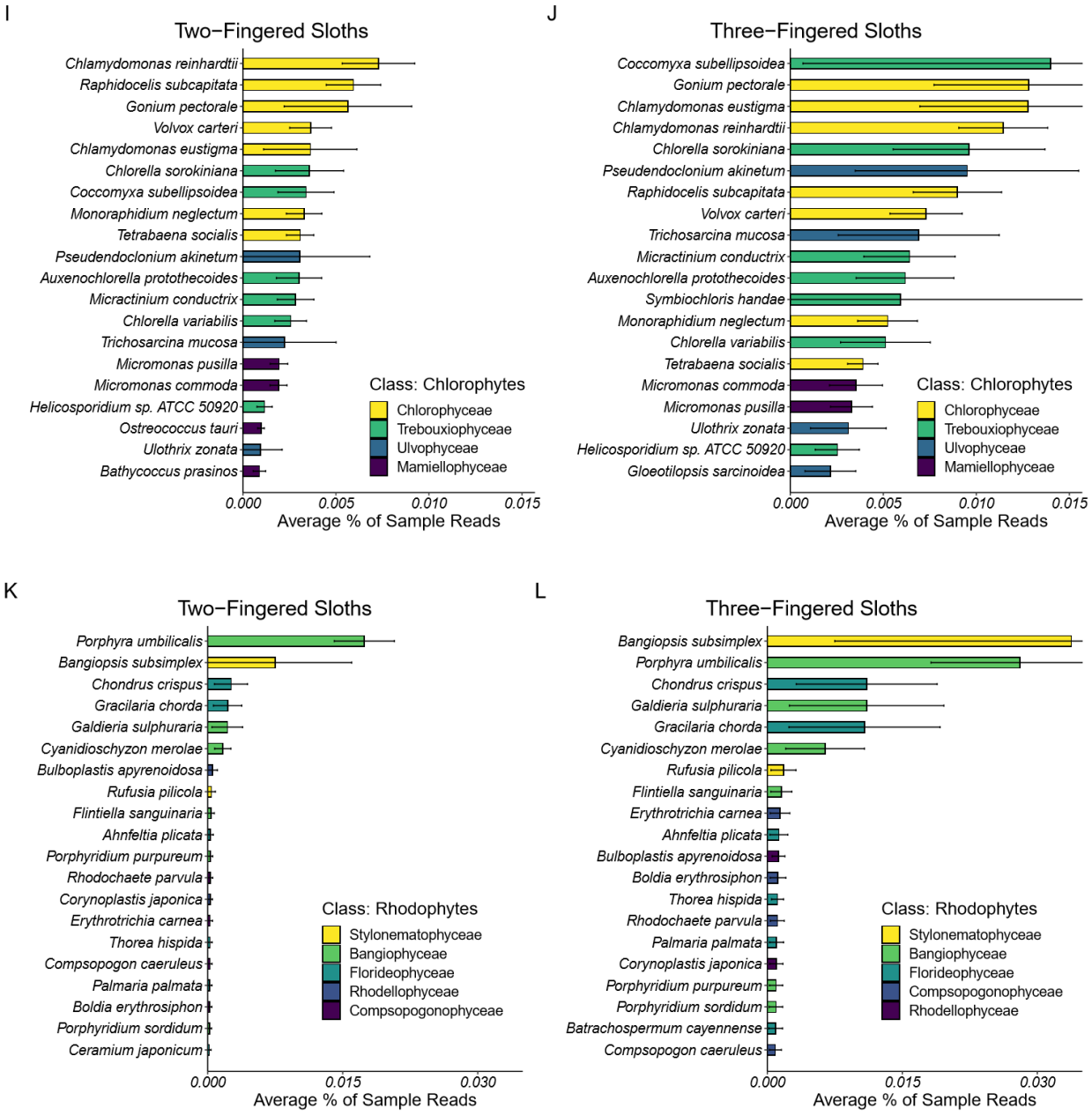


Figure 3. The average percentage of total sample reads (error bars indicate standard deviation) for the top 20 most abundant species of bacteria (A & B), archaea (C & D), fungi (E & F), cyanobacteria (G & H), chlorophytes (I & J), and rhodophytes (K & L) for both two- and three-fingered sloths. Composition was highly variable for cyanobacteria between three-fingered sloths, which resulted in very large standard deviation values and thus they are not shown. *Incertae sedis* denotes an assortment of taxa with uncertain or unresolved phylogenetic placement. Parentheses around a taxon in the legend indicates that it did not appear in the top 20 species for that sloth type, but it did for the other.

Table 5. Symbionts other than bacteria, archaea, fungi, green algae, and red algae that were found on both two- and three-fingered sloths. The three most common taxa/matches are listed in decreasing order of prevalence. The numbers indicate total species across all sampled sloths, excluding singletons. Phyla with asterisks contain representatives that are photosynthetic.

Phylum	Hoffmann's two-fingered sloth (*Choloepus hoffmanni*)		Brown-throated three-fingered sloth (*Bradypus variegatus*)	
	# of species	Most common species (in order)	# of species	Most common species (in order)
Acavomonidia	1	*Acavomonas peruviana*	1	*Acavomonas peruviana*
Amoebozoa	56	*Acanthamoeba castellanii* *Protostelium sp. Jena Gg-2016a* *Acytostelium subglobosum*	70	*Protostelium sp. Jena Gg-2016a* *Acanthamoeba castellanii* *Acytostelium subglobosum*
Apicomplexa	92	*Eimeria mitis* *Toxoplasma gondii* *Besnoitia besnoiti*	92	*Eimeria mitis* *Besnoitia besnoiti* *Toxoplasma gondii*
Centroheliozoa	1	*Raphidiophrys contractilis*	1	*Raphidiophrys contractilis*
Cercozoa*	27	*Plasmodiophora brassicae* *Bigelowiella natans* *Paulinella micropora*	31	*Plasmodiophora brassicae* *Paracercomonas marina* *Bigelowiella natans*
Chromerida*	3	*Vitrella brassicaformis* *Chromera velia* *Chromerida sp.* RM11	3	*Vitrella brassicaformis* *Chromera velia* *Chromerida sp.* RM11
Ciliophora	90	*Paramecium tetraurelia* *Tetrahymena thermophila* *Ichthyophthirius multifiliis*	89	*Paramecium tetraurelia* *Tetrahymena thermophila* *Stentor coeruleus*
Cryptista*	26	*Guillardia theta* *Cryptomonas curvata* *Teleaulax amphioxeia*	32	*Guillardia theta* *Cryptomonas curvata* *Hemiselmis andersenii*
Dinozoa*	39	*Symbiodinium microadriaticum* *Symbiodinium sp.* clade C *Karlodinium veneficum*	60	*Symbiodinium microadriaticum* *Heterocapsa triquetra* *Lepidodinium chlorophorum*
Euglenozoa*	106	*Trypanosoma cruzi* *Bodo saltans* *Leptomonas pyrrhocoris*	115	*Trypanosoma conorhini* *Trypanosoma cruzi* *Bodo saltans*

Foraminifera	3	*Reticulomyxa filosa* *Ovammina opaca* *Hyalinea balthica*	7	*Reticulomyxa filosa* *Ovammina opaca* *Hyalinea balthica*
Glaucophyta*	10	*Cyanophora tetracyanea* *Gloeochaete wittrockiana* *Cyanophora paradoxa*	11	*Cyanophora sudae* *Cyanophora tetracyanea* *Gloeochaete wittrockiana*
Haptophyta*	20	*Emiliania huxleyi* *Chrysochromulina sp.* CCMP291 *Pavlova lutheri*	35	*Emiliania huxleyi* *Chrysochromulina sp.* CCMP291 *Pavlova lutheri*
Jakobea	7	*Andalucia godoyi* *Stygiella incarcerata* *Seculamonasis ecuadoriens*	7	*Andalucia godoyi* *Stygiella incarcerata* *Seculamonasis ecuadoriens*
Loukozoa	38	*Tritrichomonas foetus* *Trichomonas vaginalis* *Giardia intestinalis*	40	*Tritrichomonas foetus* *Trichomonas vaginalis* *Giardia intestinalis*
Obazoa	14	*Thecamonas trahens* *Salpingoeca rosetta* *Monosiga brevicollis*	20	*Salpingoeca rosetta* *Thecamonas trahens* *Monosiga brevicollis*
Ochrophyta*	207	*Thalassiosira oceanica* *Ectocarpus siliculosus* *Aureococcus*	340	*Thalassiosira oceanica* *Ectocarpus siliculosus* *Aureococcus*
Olpidiomycota	2	*Olpidium bornovanus* *Olpidium brassicae*	2	*Olpidium bornovanus* *Olpidium brassicae*
Oomycota	49	*Phytophthora palmivora* *Aphanomyces astaci* *Phytophthora megakarya*	98	*Phytophthora palmivora* *Phytophthora megakarya* *Aphanomyces astaci*
Percolozoa	15	*Naegleria gruberi* *Tsukubamonas globosa* *Pharyngomonas kirbyi*	18	*Naegleria gruberi* *Pharyngomonas kirbyi* *Stachyamoeba lipophora*
Perkinsozoa	3	*Perkinsus marinus* *Perkinsus olseni* *Perkinsus chesapeaki*	3	*Perkinsus marinus* *Perkinsus olseni* *Perkinsus chesapeaki*
Placidozoa	1	*Proteromonas lacertae*	1	*Proteromonas lacertae*
Radiolaria	2	*Sticholonche zanclea* *Lithomelissa setosa*	4	*Sticholonche zanclea* *Lithomelissa setosa* *Collozoum inerme*
Stramenopiles	18	*Blastocystis sp.* subtype 4 *Blastocystis hominis* *Blastocystis sp.* subtype 1	27	*Blastocystis hominis* *Blastocystis sp.* subtype 4 *Blastocystis sp.* subtype 1
Streptophyta*	1	*Koliella corcontica*	2	*Koliella corcontica* *Raphidonema nivale*
Telonemia	1	*Telonema subtile*	1	*Telonema subtile*

DISCUSSION

Studies on the sloth hair microbiome are scarce and the microbes identified on sloths by molecular means have only been done through limited amplicon sequencing (for fungi and algae, Higginbotham et al., 2014; Suutari et al., 2010), focusing on a minimal number of species (in the case of *B. variegatus*, the single green alga, *T. welckeri*) instead of characterizing the whole community of associated microorganisms. This is the first attempt to clarify the diversity of microorganisms on both two- and three-fingered sloths using next-generation sequencing. Using whole community shotgun metagenomics, I have greatly increased the known diversity of microorganisms in the sloth fur ecosystem.

Interestingly, *T. welckeri*, the previously identified sole green alga found on *B. variegatus* was not identified among our sequences by Kaiju matches to the NCBI nr_euk database (and the *T. welckeri* sequence is indeed in the database). Perhaps the hair microbiome of sloths varies depending on location/habitat/environment; the sloths sampled in previous studies that identified *T. welckeri* were primarily from Panama and a site in the Caribbean coastal plain of northeast Costa Rica (Pauli et al., 2014; Suutari et al., 2010), which is a different habitat than the Mid Pacific coast of Costa Rica (Manuel Antonio) where samples were collected for this study. The absence of *T. welckeri* may also be due to insufficient taxonomic resolution as represented by reference sequences in the nr_euk database and/or the inability for the Kaiju method to definitively assign reads to those *T. welckeri* sequences. Nonetheless, the diversity of green algae and cyanobacteria found on both species of sloths calls into question the validity of past statements claiming that *T. welckeri* is the (only) alga responsible for brown-throated three-fingered sloths' green coloration (Aiello, 1985; Suutari et al., 2010), and that it is uniquely involved in a three-way mutualism with sloths and moths (Pauli et al., 2014; see Chapter I).

Pauli et al. (2014) have proposed that sloths are involved in an evolutionary trade-off in which they risk their lives, descending to the ground to defecate, in order to preserve this sloth-moth-algae tripartite mutualism. This study also speculated that sloths benefit by eating *T. welckeri* that grows in their fur, that moths benefit by laying their eggs in sloth feces when the sloth defecates at the base of a tree, and that *T. welckeri* benefits by receiving essential nutrients (particularly nitrogen) from moth defecation in sloth fur. There are many problems with this proposal (see "Algal Benefits" and "Sloth Moths" sections in Chapter I), most important of which is that morphology was used to designate algal species, which is not a definitive method to identify species, especially given how this taxon is often morphologically cryptic and under-studied (Dudgeon et al., 2017). With 1,150 species of green algae and cyanobacteria identified by whole community metagenomic sequencing, it is likely that the simple proposed three-way mutualism and the supposed coevolution of *T. welckeri* and *B. variegatus* (Fountain et al., 2017), are far more complex. While the tripartite mutualism and coevolution of sloth and alga cannot be ruled out, it will be crucial to determine: (i) if the presence of *T. welckeri* is determined by geographic location, (ii) if *T. welckeri* is found in sloths' stomach contents, (iii) if it grows environmentally (since sloths don't lick themselves), (iv) which of the 1000+ species of algae are obligate, and (v) how these species are transmitted to sloths.

Compositional differences in the hair microbiome of the two sloth species are subtle, but statistically significant. Three-fingered sloths have a higher proportion of photosynthetic microbes in their fur. Hair microbiomes differ by sloth species but not by location where the hair was sampled on the sloth (head or shoulder). This lack of a statistical significance between head and shoulder samples suggests sufficient dispersal and mixing of hair microbes between head and shoulder locations, which may be more more pronounced during the wet season when the

sloths' coat is wet and might facilitate mixing of microbes across the sloths' body. The differences between sloth species could be due to species-specific morphologies of sloth hair (Aiello, 1985) that may have the potential to shape the extent and composition of symbiotic growth. Three-fingered sloth hair has transverse cracks that increase in number and depth as sloths age while two-fingered sloths have vertical grooves that do not appear to absorb as much water (Figure 4 of Chapter I; Aiello, 1985; Wujek & Cocuzza, 1986). Future research should aim to understand if such hair cracks/grooves facilitate algal/microbial growth, if the microbial composition changes on sloth hair as the cracks and grooves develop and deepen with age, and whether they have co-evolved with the associated microbes.

Differences in the sloth hair microbiome between *C. hoffmanni* and *B. variegatus* could also be attributed in part to differences in their behavior. *C. hoffmanni* are nocturnal, while *B. variegatus* are cathemeral (neither nocturnal nor diurnal, but irregularly active night and day; Sunquist & Montgomery, 1973). This could affect the microbiomes' access to sunlight; *B. variegatus* are more likely to be out during the day and more sunlight may reach the microbes in their fur, while *C. hoffmanni* are generally asleep and shaded by the tree canopy during the day (Sunquist & Montgomery, 1973). *B. variegatus* also exhibit basking behavior during the day (Goodwin, 2014), which could increase the temperature and decrease the moisture content in the sloth hair ecosystem and influence microbial community composition. These characteristics of the *B. variegatus* fur environment could explain the higher proportion of photosynthetic microbes in their fur microbiome compared to *C. hoffmanni*.

Two- and three-fingered sloths had similar degrees of microbial biodiversity, as measured by the Inverse Simpson and Shannon indices (Chernov et al., 2015; Shannon, 1948; Simpson, 1949; Table 2). Whether or not the diversity of the hair microbiome of these two species of

sloths is significantly different depends on the diversity index used, but the microbiome of *C. hoffmanni* fur is more diverse based on the Inverse Simpson metric. While both indices take into consideration species richness and evenness, the Shannon index is primarily determined by the evenness of species abundances while the Inverse Simpson indices are indicators of the dominance of one or a couple species (Chernov et al., 2015). The inconsistencies in the level of evenness and the presence of one or a couple dominant species across sloth type and microbial taxonomic grouping (Figure 3) could explain the differences observed between Simpson/Inverse Simpson diversity vs. Shannon diversity. The diversity of the sloth fur microbiome is in the range of those observed for soil (Abraham et al., 2020; Castañeda & Barbosa, 2017; Choi et al., 2017; García-Salamanca et al., 2012; Gastauer et al., 2019) and plant phyllospheres (Copeland et al., 2015). The diversity is comparable to the skin microbiome of bats, one of the only land mammals whose skin microbiome has been sequenced (Shannon diversity index estimated to be ~5.2 for bat skin vs. ~5.1-5.2 for sloth hair; Avena et al., 2016), and is more diverse than the human skin microbiome (Shannon diversity index estimated to be ~0.9–2.6; Grice et al., 2009). These comparisons must be taken hesitantly, however, considering that these skin microbiome studies have focused solely on the prokaryotic diversity and used amplicon-based approaches instead of whole-community shotgun sequencing. This work represents the first hair microbiome study to be performed, so comparisons with other mammalian hair microbiomes is not possible. The diversity of algae in sloth hair remains unique, however, with the only other known mammals with algae in their fur being polar bears in zoos (Lewin & Robinson, 1979) and manatees whose algae grows more so on their skin than fur (Bledsoe et al., 2006). Unfortunately, the Shannon index values for the only gut microbiome study of sloths were not reported (Dill-McFarland et al., 2015), so I am not able to compare them to the hair microbiome.

Species designations through bioinformatic database matching are intrinsically limited by the database of known genetic diversity. While the NCBI nr_euk database is arguably the most comprehensive reference database for metagenomics, the microbial taxa in sloth hair may be largely uncharacterized, as indicated by the substantial fraction (≥58%) of unclassified read sequences in our dataset. This problem of "unknown unknowns" suggests that at least some species designations may be flawed, and more robust phylogenetic sequence-based inference methods using multiple loci (Luo et al., 2018; Zhang et al., 2014) and/or read-assembly methods (Bowers et al., 2017; Castelle & Banfield, 2018; Olm et al., 2020) may be needed to resolve new taxa. This substantial fraction of the reads that are unclassified likely indicates that there is novel genetic diversity to be analyzed in the sloth fur microbiome that is not represented in the NCBI non-redundant database of known sequences.

Of the species that were identified, however, one of the most prominent species of bacteria, *Enterococcus faecium,* is a commensal or parasitic bacterium in the gastrointestinal tracts of humans and other animals, and is the second most common cause of hospital-acquired infections (Schaberg et al., 1991). *E. faecium* and an unclassified Acidobacterium were 2-3 times more prevalent than other bacterial species, suggesting that the bacterial community on sloth hair is quite uneven, with a couple of dominant species and many species in much lower abundances. Archaeal species show a similar "spike+long tail" trend, with a few dominant species and many less abundant species. The most prevalent species was an unclassified archaeon; the second most prevalent species of archaea identified for *C. hoffmanni* was *Candidatus Nitrocosmicus oleophilus,* which is a terrestrial species found in soil and sediment (Jung et al., 2016) and for *B. variegatus,* an unclassified *Thermoplasmata* archaeon.

The yeast *Saccharomycodes ludwigii* was by far the most prominent species match of fungi for both species of sloths examined; all other fungal species have much lower abundances. This species is a wine-spoilage yeast that has also been used in experiments on other fermented beverages and on the production of aroma compounds (Tavares et al., 2018). *S. ludwigii* is very tolerant of high sulphite concentrations (Stratford et al., 1987). This species performs ammonia assimilation during ammonia limitation using glutamine synthetase and glutamate synthase (Johnson & Brown, 1974). *S. ludwigii* ferments and produces acetoin and ethyl acetate, which is a common characteristic of yeasts, but also has an unusually high production of isobutanol (Romano et al., 1999).

Nine of the 16 genera of fungi identified by culture- and amplicon-based surveys of fungi found in the fur of the three-fingered sloth, *B. variegatus* were found in our datasets for both *B. variegatus* and *C. hoffmanni* (namely, *Arthrinium*, *Colletotrichum*, *Cytospora*, *Fusarium*, *Lasiodiplodia*, *Leptosphaeria*, *Penicillium*, *Pestalotiopsis*, and *Phaeoacremonium*) (Higginbotham et al., 2010). Of the remaining seven genera of fungi, four are represented at the family level in our dataset (Bionectriaceae, Botryosphaeriaceae, Xylariaceae, and Hypocreaceae) while three are not (Montagnulaceae, Cephalotheceae, and Amphisphaeriaceae; Higginbotham et al., 2010). This suggests that perhaps some fungi are transient on sloth fur, or that the fungal microbiome varies depending on geographic location, given that Higginbotham et al. (2010) sampled sloths exclusively in Soberanía National Park in Panama. It is clear that shotgun metagenomic techniques give a deeper representation of the diversity of fungal species, considering that 16 genera were identified using culturing and amplicon-sequencing methods while 808 genera were identified here using whole-community metagenomics.

The top 20 chlorophyte species for *C. hoffmanni* and *B. variegatus* were much more even than those for bacteria, archaea, and fungi. These species represent the top four chlorophyte classes: Chlorophyceae, Trebouxiophyceae, Ulvophyceae, and Mamiellophyceae. These results are just a snapshot of the total chlorophyte diversity on sloth hair as there are 434 identified species on *C. hoffmanni* and 578 species on *B. variegatus*. The single-celled model green alga, *Chlamydomonas reinhardtii*, was the most common chlorophyte match for *C. hoffmanni*. Although *C. reinhardtii* is typically found in temperate soils and biological soil crusts, it's natural ecology is still poorly understood (Sasso et al., 2018); it possible that sloths acquire this alga when in contact with soil while defecating at the base of a tree. *Raphidocelis subcapitata*, a freshwater alga, is the second most common green alga match on *C. hoffmanni*. *Gonium pectorale*, the third most prevalent green alga match for *C. hoffmanni* and second most prevalent on *B. variegatus*, is also found in freshwater (lakes, ponds, and rivers), and is known to be a cosmopolitan and multicelluar species distantly related to *C. reinhardtii* (Pentecost, 2002). *Coccomyxa subellipsoidea*, the top hit of green algae on *B. variegatus*, is a worldwide subaerial and freshwater species that can tolerate polar environments and can sometimes be found as a lichen photobiont (Acton, 1909; Blanc et al., 2012; Darienko et al., 2015), and thus is a possible candidate to form mutualistic associations with fungi in sloth fur. *Chlamydomonas eustigma* is the third most common chlorophyte on *B. variegatus*; little is known about this species except that it is a distant *Chlamydomonas reinhardtii* relative and is acidophilic (Hirooka et al., 2017).

Cyanobacteria on sloth fur do not appear to be heavily dominated by one or two species like other sloth hair-associated bacteria. The top twenty species shown in Figure 3 are a small portion of the 440 and 572 species of cyanobacteria found on *C. hoffmanni* and *B. variegatus*, respectively. The top 3 species matches of cyanobacteria on *C. hoffmanni* were cyanobacterium

TDX16, *Hassallia byssoidea* (a terrestrial, filamentous Nostoc sp.), and an undescribed Oscillatoriacean cyanobacterium. The top three cyanobacteria species on *B. variegatus* were *Aliterella atlantica* (a known marine species), *Synechocystis* sp. PCC 7509, and *Chroococcidiopsis cubana* (a freshwater species). Because these are all model cyanobacteria and since cyanobacteria remain, on the whole, taxonomically poorly-resolved, these species hits may be an artifact of the incompleteness of the NCBI nr_euk database and/or false positive assignments using the Kaiju method with this database. The top three species hits of rhodophytes for both *C. hoffmanni* and *B. variegatus* were *Porphyra umbilicalis* (described as a coldwater seaweed), *Bangiopsis subsimplex* (marine red alga), and *Chondrus crispus* (intertidal seaweed). These are likely not what is truly growing on sloth fur since they are seaweeds, which remain poorly resolved taxonomically (Yoon et al., 2006) and suggests that there may be new species of rhodophytes on sloth fur whose closest matches in the NCBI database are marine seaweeds.

The diversity of known groups of symbionts on sloth fur has increased. We were previously aware of a handful of species from the groups Euglenozoa, Amoebozoa, Cercozoa, Apicomplexa, Dynophyceae, and Ciliophora (see Table 4 in Chapter I; Gilmore et al., 2001; Suutari et al., 2010). Our whole community shotgun metagenomic sequencing efforts have expanded the known diversity of species in these groups, as well as identified new sloth fur symbionts from 20 more phyla.

Many parasitic protists have been identified in the sloth hair microbiome. Ninety-two species of parasites in the class Trypanasomatidae, which include trypanosomes such as *Trypanosoma cruzi* (which causes Chagas disease), and *Leishmania major* (which causes zoonotic cutaneous leishmaniasis) are found on both two- and three-fingered sloths. Well-known human parasites, such as the brain-eating amoeba, *Naegleria fowleri*, *Giardia intestinalis*,

Toxoplasma gondii, and *Trichomonas vaginalis*, are also found in sloth hair. It is unknown whether these parasites infect sloths. They may not harm the sloth when in low abundance in their hair, but could potentially become parasitic to sloths when immunocompromised. Sloths may be accidental hosts and not reservoirs, having acquired the parasite but with a low infection rate, which is common for some blood parasites like *T. cruzi* (Shaw, 1985). While the extent to which these parasites infect sloths is unknown, many of these species have been found in the analysis of sloth blood (reviewed by Gilmore et al., 2001; Herrer & Chistensen, 1980; Shaw, 1985; Travi et al., 1989). Regardless, the diversity of parasitic symbionts in sloth fur suggests that human interaction with sloths should be minimized, not only for the sloths' well-being, but to protect humans from contracting a life-threatening parasite.

The vast diversity of species on sloth fur suggests that previous studies may have been premature in making conclusions about the ecology and behavior of sloths in regards to the sloth fur ecosystem. The validity of taxonomic assignments described here requires confirmation using additional phylogenetic and phylogenomic comparison methods. Efforts to construct genome drafts from the whole community metagenomic data may aid in identifying and describing new microbial species (Iverson et al., 2012; Parks et al., 2017; Sieber et al., 2018). Such work should be paired with culturing methods if possible to work towards a description of new species. Once this baseline of biodiversity on sloth fur has been established, we will be better prepared to address more targeted ecological questions. Answering basic ecological questions will provide insights into how the sloth fur ecosystem might be specific to sloth species, geographic location, and season, whether there is coevolution between sloths and their fur microbes, whether fur microbes are mutualistic, commensal, or parasitic, and how best to care for sloths in rehabilitation facilities.

Determining the diversity of the sloth fur ecosystem using shotgun metagenomic sequencing is the first step in helping us understand what members of the microbiome may be beneficial or harmful to the sloth host, and which may impact the sloth's fitness. Gene function analysis of the metagenome data remains to be performed, and may help us understand microbial contributions to sloth fitness and fur ecosystem functions. Discovering microbiome-associated predictive metrics of sloth health will be just as crucial as elucidating the ecology of parasitic symbionts for sloth conservation efforts. The knowledge that sloths carry so many life-threatening human parasites in their fur is helpful to sloth conservation if used as a means to deter the public from keeping sloths as pets or handling sloths to take photos with them as part of the "sloth selfie" trade. We must strive to understand how these parasites are transmitted, and if they can infect the sloth by being in their hair or if the sloths are simply carriers. Understanding how microbiome dysbiosis is linked to sloth disease, and if certain microbial species might protect the sloth from being susceptible to disease, are critical topics for future study.

CONCLUSIONS

1) Whole community metagenomic sequencing has expanded our understanding of the sloth fur ecosystem, revealing the extent of microbial species diversity on *B. variegatus* and *C. hoffmanni* fur. The rich diversity of microbes on sloth fur (especially of algae) challenges preconceived ideas about what causes sloths to be green and suggests that sloth fur may harbor undescribed biodiversity.
2) The fur microbiomes of *B. variegatus* and *C. hoffmanni* differ in species composition. *B. variegatus* have proportionally more photosynthetic microbes in their fur than *C. hoffmanni*. There is no statistical difference between the microbes found on hair from the

head or shoulder of a sampled sloth, however. Diversity indices indicate that species diversity of the two sloth species' microbiomes is similar and comparable to published estimates of soil and phyllosphere microbial diversity.

3) While the algae and fungi on sloth fur has gained the most attention in the literature, sloth-associated bacteria and protists warrant further study, especially considering the potential for these microbes to be parasitic and to infect the sloth and humans that interact with sloths. Exploring the capability of parasitic bacteria and protists in sloth fur to infect their host should be a priority in future sloth conservation studies.
4) Sloth conservation efforts should take the diversity of sloth hair microbes into consideration since the microbes living and growing in sloth fur have the potential to protect the sloth from pathogens and also infect the sloth to cause disease. Understanding the modes of transmission, the pathogenicity, and the community ecology of these microbes is essential to determine the role of the hair microbes in sloth health.

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APPENDIX

```
library(tidyverse)
library(reshape2)    # https://seananderson.ca/2013/10/19/reshape/
library(vegan)       # uses adonis for PERMANOVA
library(ggplot2)
library(ggthemes)
library(patchwork)
library(matrixStats)
library(viridis)

##metadata file
meta_path <- "Metadata2019.csv"   # path to the meta-data file
##data folder
data_path <- "starting-kaiju-output-data"   # path to the data folder
##data files
files <- dir(data_path, pattern = "*list.tsv") # get file names
##output directory
setwd("processed-data")

##load metadata with data-ordered and
##grouped appropriately for NMDS/Permanova/split-plot analyses
meta <- read_csv(meta_path)
## this will also used as "factors" file for PERMANOVA analysis
# index	file	name	type	location	season
# 1	SHLe29.kaiju.out	Cher	2F	Head	dry
# 2	SHLe33.kaiju.out	Freddie	2F	Head	dry
# 3	SHLe32.kaiju.out	Gwen	2F	Head	dry
# 4	SHLe27.kaiju.out	Judy	2F	Head	dry
# 5	SHLe15.kaiju.out	Madonna	2F	Head	dry
# 6	SHLe30.kaiju.out	Cher	2F	Shoulder	dry
# 7	SHLe34.kaiju.out	Freddie	2F	Shoulder	dry
# 8	SHLe31.kaiju.out	Gwen	2F	Shoulder	dry
# 9	SHLe28.kaiju.out	Judy	2F	Shoulder	dry
# 10	SHLe16.kaiju.out	Madonna	2F	Shoulder	dry
# 11	SHLe21.kaiju.out	Aladdin	3F	Head	dry
# 12	SHLe22.kaiju.out	Buzz	3F	Head	dry
# 13	SHLe17.kaiju.out	Esperanza	3F	Head	dry
# 14	SHLe19.kaiju.out	Merlin	3F	Head	dry
# 15	SHLe24.kaiju.out	Shuri	3F	Head	dry
# 16	SHLe23.kaiju.out	Tarzan	3F	Head	dry
# 17	SHLe1.kaiju.out	Aladdin	3F	Shoulder	dry
# 18	SHLe2.kaiju.out	Buzz	3F	Shoulder	dry
# 19	SHLe18.kaiju.out	Esperanza	3F	Shoulder	dry
# 20	SHLe20.kaiju.out	Merlin	3F	Shoulder	dry
# 21	SHLe4.kaiju.out	Shuri	3F	Shoulder	dry
```

```
# 22     SHLe3.kaiju.out         Tarzan 3F        Shoulder         dry

##load Kaiju data
#from:
#https://serialmentor.com/blog/2016/6/13/reading-and-combining-many-tidy-data-files-in-R
data <- files %>%
  map(function(x) read_tsv(file.path(data_path, x))) %>%
  reduce(rbind)

## create new data table for splitting taxonomic names
new <- array(dim = c(nrow(data), 7))

##loop through each row in original data file
for (i in 1:nrow(data)){
  #temporarily define 'x' as the un-split string
  x <- data$taxon_name[i]
  #split string by semicolon
  split_taxa <- strsplit(x, ';')
  #put the new seven subdivided names into each row of 'new'
  new[i,] <- split_taxa[[1]]
}

##name the columns of 'new'
colnames(new) <-  c('Superkingdom','Phylum','Class','Order','Family','Genus','Species')

##create 'new_total_data' by binding new onto the original data and merging with metadata
#also sort by index then name of sloth then type-location then season
#(to match metadata row order)
new_total_data <- arrange(merge(meta, cbind(data,new), by="file"),
                          index, name, type, location, season)
##convert index to a string with 2 digits
##so things will sort lexigraphically (01,02,03,...)
new_total_data$index <- sprintf("%02d", new_total_data$index)
## create fileID column; merge other columns of metadata to create an ordered label
new_total_data <- new_total_data %>%
                separate(file, "fileID", extra="drop", remove=FALSE) %>%
                unite(label, c("index","fileID", "name","type", "location", "season"),
                      sep=".", remove=FALSE)

## get filenames and set up taxa and output files
file_names <- unique(new_total_data[,'file'])
taxa <- c('superkingdom','phylum','class','order','family','genus','species')
##output file designations
outfiletaxa <- paste("SHLe-kaiju-sicklereads-taxoncounts", taxa, "tab", sep='.')
outfilereads <- paste("SHLe-kaiju-sicklereads-readcounts", taxa, "tab", sep='.')
outfilereadsnorm <- paste("SHLe-kaiju-sicklereads-readcountsNORM", taxa, "tab", sep='.')
```

```
outfiletaxaagg <- paste("SHLe-kaiju-sicklereads-taxoncounts-aggregated", taxa, "tab",
                    sep='.')
outfilereadsagg <- paste("SHLe-kaiju-sicklereads-readcounts-aggregated", taxa, "tab",
                     sep='.')
outfilereadsaggnorm <- paste("SHLe-kaiju-sicklereads-readcountsNORM-aggregated", taxa,
                        "tab", sep='.')
outfiletotalreads <- "SHLe-kaiju-sicklereads-TOTALreadcounts.tab"

### TOTAL SAMPLE READ COUNTS NORMALIZATION
tt <- as.data.frame(new_total_data %>% group_by(label) %>% summarize(total=sum(reads)))
write_tsv(tt,path=outfiletotalreads)
##normalize
new_total_data <- merge(new_total_data,tt)
new_total_data$normreads <- new_total_data$reads / new_total_data$total

#####################################
### SUPERKINGDOM LEVEL READ COUNTS ###
#####################################
temp <- dcast(melt(new_total_data %>% group_by(label, Superkingdom) %>%
               summarize(readcount=sum(reads)), id.vars=c("Superkingdom", "label")),
                     Superkingdom ~ label)
tempnorm <- dcast(melt(new_total_data %>% group_by(label, Superkingdom) %>%
                   summarize(readcount=sum(normreads)),
                   id.vars=c("Superkingdom", "label")), Superkingdom ~ label)
##2F dry season
sub2 <- temp %>% select(Superkingdom, contains("2F")) %>%
             select(Superkingdom, contains("dry"))
sub2norm <- tempnorm %>% select(Superkingdom, contains("2F")) %>%
                   select(Superkingdom, contains("dry"))
##3F dry season
sub3 <- temp %>% select(Superkingdom, contains("3F")) %>%
             select(Superkingdom, contains("dry"))
sub3norm <- tempnorm %>% select(Superkingdom, contains("3F")) %>%
                   select(Superkingdom, contains("dry"))

##calculate aggregated sums and stats for 2F dry and 3F dry groups
sub2 %>% select(-Superkingdom) %>% rowSums(na.rm=TRUE) -> temp$TwoF_Dry
sub2norm %>% select(-Superkingdom) %>% rowSums(na.rm=TRUE) -> tempnorm$TwoF_Dry
sub3 %>% select(-Superkingdom) %>% rowSums(na.rm=TRUE) -> temp$ThreeF_Dry
sub3norm %>% select(-Superkingdom) %>% rowSums(na.rm=TRUE) ->
tempnorm$ThreeF_Dry
temp <- temp %>% mutate(TwoF_Dry.freq=TwoF_Dry/sum(TwoF_Dry),
                    ThreeF_Dry.freq=ThreeF_Dry/sum(ThreeF_Dry)) %>%
               mutate_all(~replace(., is.na(.), 0)) #remove NAs
tempnorm <- tempnorm %>% mutate(TwoF_Dry.freq=TwoF_Dry/sum(TwoF_Dry),
                        ThreeF_Dry.freq=ThreeF_Dry/sum(ThreeF_Dry)) %>%
```

```
                  mutate_all(~replace(., is.na(.), 0)) #remove NAs
agg <- temp %>% select(Superkingdom, TwoF_Dry, ThreeF_Dry, TwoF_Dry.freq,
                ThreeF_Dry.freq)
aggnorm <- tempnorm %>% select(Superkingdom, TwoF_Dry, ThreeF_Dry, TwoF_Dry.freq,
                    ThreeF_Dry.freq)

##write out aggregate data (unnormalized reads)
write_tsv(agg,path=outfilereadsagg[1])
##output will have sum of normalized counts for each sloth,
##totaled for 10x2F sloths or 12x3F sloths
write_tsv(aggnorm,path=outfilereadsaggnorm[1])
##write out FULL data (non transposed so it can be read with Excel) with taxa in rows
#(Excel limit is ~1 million rows but 16,000 columns)
write_tsv(temp,path=outfilereads[1])
write_tsv(tempnorm,path=outfilereadsnorm[1])

################################
### PHYLUM LEVEL READ COUNTS ###
################################
temp <- dcast(melt(new_total_data %>% group_by(label, Superkingdom, Phylum) %>%
              summarize(readcount=sum(reads)) %>%
              unite(superkingdom_PHYLUM, Superkingdom, Phylum, sep=";"),
                  id.vars=c("superkingdom_PHYLUM", "label")),
                  superkingdom_PHYLUM ~ label)
tempnorm <- dcast(melt(new_total_data %>% group_by(label, Superkingdom, Phylum) %>%
                summarize(readcount=sum(normreads)) %>%
                unite(superkingdom_PHYLUM, Superkingdom, Phylum, sep=";"),
                    id.vars=c("superkingdom_PHYLUM", "label")),
                    superkingdom_PHYLUM ~ label)
##2F dry season
sub2 <- temp %>% select(superkingdom_PHYLUM, contains("2F")) %>%
            select(superkingdom_PHYLUM, contains("dry"))
sub2norm <- tempnorm %>% select(superkingdom_PHYLUM, contains("2F")) %>%
                  select(superkingdom_PHYLUM, contains("dry"))
##3F dry season
sub3 <- temp %>% select(superkingdom_PHYLUM, contains("3F")) %>%
            select(superkingdom_PHYLUM, contains("dry"))
sub3norm <- tempnorm %>% select(superkingdom_PHYLUM, contains("3F")) %>%
                  select(superkingdom_PHYLUM, contains("dry"))

##calculate aggregated sums and stats for 2F dry, 3F dry, and 3F wet groups
sub2 %>% select(-superkingdom_PHYLUM) %>% rowSums(na.rm=TRUE) ->
temp$TwoF_Dry
sub2norm %>% select(-superkingdom_PHYLUM) %>% rowSums(na.rm=TRUE) ->
tempnorm$TwoF_Dry
```

```
sub3 %>% select(-superkingdom_PHYLUM) %>% rowSums(na.rm=TRUE) ->
temp$ThreeF_Dry
sub3norm %>% select(-superkingdom_PHYLUM) %>% rowSums(na.rm=TRUE) ->
tempnorm$ThreeF_Dry
temp <- temp %>% mutate(TwoF_Dry.freq=TwoF_Dry/sum(TwoF_Dry),
                  ThreeF_Dry.freq=ThreeF_Dry/sum(ThreeF_Dry)) %>%
              mutate_all(~replace(., is.na(.), 0)) #remove NAs
tempnorm <- tempnorm %>% mutate(TwoF_Dry.freq=TwoF_Dry/sum(TwoF_Dry),
                        ThreeF_Dry.freq=ThreeF_Dry/sum(ThreeF_Dry)) %>%
                    mutate_all(~replace(., is.na(.), 0)) #remove NAs
agg <- temp %>% select(superkingdom_PHYLUM, TwoF_Dry, ThreeF_Dry, TwoF_Dry.freq,
                  ThreeF_Dry.freq)
aggnorm <- tempnorm %>% select(superkingdom_PHYLUM, TwoF_Dry, ThreeF_Dry,
TwoF_Dry.freq,
                        ThreeF_Dry.freq)

##write out aggregate data (unnormalized reads)
write_tsv(agg,path=outfilereadsagg[2])
##output will have sum of normalized counts for each sloth,
##totaled for 10x2F sloths or 12x3F sloths
write_tsv(aggnorm,path=outfilereadsaggnorm[2])
##write out FULL data (non transposed so it can be read with Excel) with taxa in rows
#(Excel limit is ~1 million rows but 16,000 columns)
write_tsv(temp,path=outfilereads[2])
write_tsv(tempnorm,path=outfilereadsnorm[2])

#########################################################################
### SPECIES LEVEL READ COUNTS - used for all susequent analyses ###
#########################################################################
temp <- dcast(melt(new_total_data %>%
               group_by(label, Superkingdom, Phylum, Class, Order, Family, Genus,
                     Species) %>% summarize(readcount=sum(reads)) %>%
               unite(superkingdom_phylum_class_order_family_genus_SPECIES,
                    Superkingdom, Phylum, Class, Order, Family, Genus, Species,
                    sep=";"),
                    id.vars=c("superkingdom_phylum_class_order_family_genus_SPECIES",
                          "label")),
                    superkingdom_phylum_class_order_family_genus_SPECIES ~ label)
tempnorm <- dcast(melt(new_total_data %>%
                  group_by(label, Superkingdom, Phylum, Class, Order, Family, Genus,
                        Species) %>% summarize(readcount=sum(normreads)) %>%
                  unite(superkingdom_phylum_class_order_family_genus_SPECIES,
                       Superkingdom, Phylum, Class, Order, Family, Genus, Species,
                       sep=";"),
                  id.vars=c("superkingdom_phylum_class_order_family_genus_SPECIES",
                        "label")),
```

```
                    superkingdom_phylum_class_order_family_genus_SPECIES ~ label)
#2F dry season
sub2 <- temp %>% select(superkingdom_phylum_class_order_family_genus_SPECIES,
                  contains("2F")) %>%
              select(superkingdom_phylum_class_order_family_genus_SPECIES,
                  contains("dry"))
sub2norm <- tempnorm %>%
select(superkingdom_phylum_class_order_family_genus_SPECIES,
                        contains("2F")) %>%
                    select(superkingdom_phylum_class_order_family_genus_SPECIES,
                        contains("dry"))
#3F dry season
sub3 <- temp %>% select(superkingdom_phylum_class_order_family_genus_SPECIES,
                  contains("3F")) %>%
              select(superkingdom_phylum_class_order_family_genus_SPECIES,
                  contains("dry"))
sub3norm <- tempnorm %>%
select(superkingdom_phylum_class_order_family_genus_SPECIES,
                        contains("3F")) %>%
                    select(superkingdom_phylum_class_order_family_genus_SPECIES,
                        contains("dry"))

##calculate aggregated sums and stats for 2F dry, 3F dry, and 3F wet groups
sub2 %>% select(-superkingdom_phylum_class_order_family_genus_SPECIES) %>%
      rowSums(na.rm=TRUE) -> temp$TwoF_Dry
sub2norm %>% select(-superkingdom_phylum_class_order_family_genus_SPECIES) %>%
         rowSums(na.rm=TRUE) -> tempnorm$TwoF_Dry
sub3 %>% select(-superkingdom_phylum_class_order_family_genus_SPECIES) %>%
      rowSums(na.rm=TRUE) -> temp$ThreeF_Dry
sub3norm %>% select(-superkingdom_phylum_class_order_family_genus_SPECIES) %>%
      rowSums(na.rm=TRUE) -> tempnorm$ThreeF_Dry
temp <- temp %>% mutate(TwoF_Dry.freq=TwoF_Dry/sum(TwoF_Dry),
                    ThreeF_Dry.freq=ThreeF_Dry/sum(ThreeF_Dry)) %>%
             mutate_all(~replace(., is.na(.), 0)) #remove NAs
tempnorm <- tempnorm %>% mutate(TwoF_Dry.freq=TwoF_Dry/sum(TwoF_Dry),
                        ThreeF_Dry.freq=ThreeF_Dry/sum(ThreeF_Dry)) %>%
         mutate_all(~replace(., is.na(.), 0)) #remove NAs
agg <- temp %>% select(superkingdom_phylum_class_order_family_genus_SPECIES,
                   TwoF_Dry, ThreeF_Dry, TwoF_Dry.freq, ThreeF_Dry.freq)

##write out aggregate data (unnormalized reads)
write_tsv(agg,path=outfilereadsagg[7])
##write out FULL data (non transposed so it can be read with Excel) with taxa in rows
#(Excel limit is ~1 million rows but 16,000 columns)
write_tsv(temp,path=outfilereads[7])
```

```
##Create Transpose tables for running PERMANOVA etc.
##using data NORMALIZED for TOTAL READS PER SAMPLE
## USE NORMALIZED READ DATA
##2F & 3F dry season
#drop all taxa rows that have 0 counts over all sets of dry season sloths
TwoF3Fdry <- filter(full_join(sub2norm,sub3norm), tempnorm$TwoF_Dry!=0 |
                    tempnorm$ThreeF_Dry!=0) %>%
            gather(label, readcount,
                   -superkingdom_phylum_class_order_family_genus_SPECIES) %>%
            spread(superkingdom_phylum_class_order_family_genus_SPECIES, readcount) %>%
            separate(label, c("Index","FileID", "Name","Type", "Location", "Season"),
                     remove=FALSE)
#remove unassigned/unclassified reads
TwoF3Fdry <- TwoF3Fdry %>%
            select(-`unclassified;unclassified;unclassified;unclassified;unclassified;
                   unclassified;unclassified`)
#replace all NAs with zeros in prep for calculating row stats
Hist2F3Fdry <- filter(full_join(sub2norm,sub3norm), tempnorm$TwoF_Dry!=0 |
                      tempnorm$ThreeF_Dry!=0) %>%
             mutate_all(~replace(., is.na(.), 0))
#create temp matrix to calculate rowMeans and rowSds using matrixStats package
#multiply by 100 to represent as %
tempmat <- 1e2*as.matrix(Hist2F3Fdry %>%
                         select(superkingdom_phylum_class_order_family_genus_SPECIES,
                                contains("2F")) %>%
                         select(-superkingdom_phylum_class_order_family_genus_SPECIES))
Hist2F3Fdry$Mean2F <- rowMeans(tempmat)
Hist2F3Fdry$sd2F <- rowSds(tempmat)
tempmat <- 1e2*as.matrix(Hist2F3Fdry %>%
                         select(superkingdom_phylum_class_order_family_genus_SPECIES,
                                contains("3F")) %>%
                         select(-superkingdom_phylum_class_order_family_genus_SPECIES))
Hist2F3Fdry$Mean3F <- rowMeans(tempmat)
Hist2F3Fdry$sd3F <- rowSds(tempmat)
#create taxon columns from label
Hist2F3Fdry <- Hist2F3Fdry %>%
             separate(superkingdom_phylum_class_order_family_genus_SPECIES,
                      c("Superkingdom","Phylum", "Class","Order", "Family", "Genus",
                        "Species"), sep=";", remove=FALSE, extra="merge")


#############################################################################
#######
### FIGURES 1,2,3: Data Set up foStacked Bar Graphs, NMDS/PERMANOVA, & Bar Charts
###
```

```
#############################################################################
#######
##DRY SEASON ONLY - rows 1-10 = 2F ; rows 11-22 = 3F
##Generate Data Tables for Mean Bar Charts of Species
## for Top 20 Species Histograms plots
Archaea <- filter(Hist2F3Fdry, grepl("Archaea;",
                          superkingdom_phylum_class_order_family_genus_SPECIES))
BacteriaWithCyanos<- filter(Hist2F3Fdry, grepl("Bacteria;",
                            superkingdom_phylum_class_order_family_genus_SPECIES))
BacteriaNoCyanos <- filter(Hist2F3Fdry, grepl("Bacteria;",
                           superkingdom_phylum_class_order_family_genus_SPECIES)) %>%
              filter(!grepl("Bacteria;Cyanobacteria",
                        superkingdom_phylum_class_order_family_genus_SPECIES))
Cyanobacteria <- filter(Hist2F3Fdry, grepl("Bacteria;Cyanobacteria",
                            superkingdom_phylum_class_order_family_genus_SPECIES))
Chlorophyta <- filter(Hist2F3Fdry, grepl("Eukaryota;Chlorophyta",
                           superkingdom_phylum_class_order_family_genus_SPECIES))
Fungi <- filter(Hist2F3Fdry, grepl("Eukaryota;Ascomycota|Eukaryota;Basidiomycota|
                          Eukaryota;Chytridiomycota|Eukaryota;Microsporidia|
                          Eukaryota;Mucoromycota|Eukaryota;Neocallimastigomycota|
                          Eukaryota;Zoopagomycota|Eukaryota;NA;NA;NA;NA;NA;fung|
                          Eukaryota;NA;NA;NA;NA;NA;uncultured Mucoromycotina",
                          superkingdom_phylum_class_order_family_genus_SPECIES))
Rhodophyta <- filter(Hist2F3Fdry, grepl("Eukaryota;NA;Bangiophyceae|
                           Eukaryota;NA;Compsopogonophyceae|
                           Eukaryota;NA;Florideophyceae|
                           Eukaryota;NA;Rhodellophyceae|
                           Eukaryota;NA;Stylonematophyceae",
                           superkingdom_phylum_class_order_family_genus_SPECIES))
Hist2F3Fdry$OldPhylum <- Hist2F3Fdry$Phylum
## Fix "Algae" phyla = photosynthetic protists
#Cercozoa
#Chlorophyta - already phylum in nr_euk/Kaiju output
#Chromerida - already phylum in nr_euk/Kaiju output
#Cryptista
#Dinozoa
#Euglenozoa
#Glaucophyta
#Haptophyta
#Ochrophyta
#Picozoa
#Rhodophyta
#Streptophyta - already phylum in nr_euk/Kaiju output
Cercozoa <- filter(Hist2F3Fdry, grepl("Eukaryota;NA;NA;Cercomonadida;|
                          Eukaryota;NA;NA;Euglyphida;|
                          Eukaryota;NA;NA;Glissomonadida;|
```

```
                          Eukaryota;NA;NA;NA;Chlamydophryidae;|
                          Eukaryota;NA;NA;NA;Ebriidae;|
                          Eukaryota;NA;NA;NA;Mikrocytiidae;|
                          Eukaryota;NA;NA;NA;NA;Amorphochlora;|
                          Eukaryota;NA;NA;NA;NA;Bigelowiella;|
                          Eukaryota;NA;NA;NA;NA;Chlorarachnion;|
                          Eukaryota;NA;NA;NA;NA;Gymnochlora;|
                          Eukaryota;NA;NA;NA;NA;Gymnophrys;|
                          Eukaryota;NA;NA;NA;NA;Lotharella;|
                          Eukaryota;NA;NA;NA;NA;NA;Cercozoa sp. DDB-2008a|
                          Eukaryota;NA;NA;NA;NA;NA;Phaeodaria sp. OSH121|
                          Eukaryota;NA;NA;NA;NA;Partenskyella;|
                          Eukaryota;NA;NA;NA;Plasmodiophoridae;|
                          Eukaryota;NA;NA;NA;Spongomonadidae;|
                          Eukaryota;NA;NA;Phaeocystida;|
                          Eukaryota;NA;NA;Thaumatomonadida;|
                          Eukaryota;NA;NA;Vampyrellida;",
                          superkingdom_phylum_class_order_family_genus_SPECIES))
Cercozoa$Phylum <- "Cercozoa"
Cryptista <- filter(Hist2F3Fdry, grepl("Eukaryota;NA;Cryptophyta;|
                           Eukaryota;NA;NA;NA;NA;Palpitomonas;",
                           superkingdom_phylum_class_order_family_genus_SPECIES))
Cryptista$Phylum <- "Cryptista"
Dinozoa <- filter(Hist2F3Fdry, grepl("Eukaryota;NA;Dinophyceae;|
                          Eukaryota;NA;NA;NA;NA;Voromonas;",
                          superkingdom_phylum_class_order_family_genus_SPECIES))
Dinozoa$Phylum <- "Dinozoa"
Euglenozoa <- filter(Hist2F3Fdry, grepl("Eukaryota;Euglenida;|
                           Eukaryota;NA;NA;Diplonemida;|
                           Eukaryota;NA;NA;Kinetoplastida;",
                           superkingdom_phylum_class_order_family_genus_SPECIES))
Euglenozoa$Phylum <- "Euglenozoa"
Glaucophyta <- filter(Hist2F3Fdry, grepl("Eukaryota;NA;Glaucocystophyceae;",
                         superkingdom_phylum_class_order_family_genus_SPECIES))
Glaucophyta$Phylum <- "Glaucophyta"
Haptophyta <- filter(Hist2F3Fdry, grepl("Eukaryota;NA;NA;Coccolithales;|
                           Eukaryota;NA;NA;Isochrysidales;|
                           Eukaryota;NA;NA;NA;NA;NA;Haptophyceae sp. W5-1|
                           Eukaryota;NA;NA;NA;NA;NA;uncultured haptophyte|
                           Eukaryota;NA;NA;NA;NA;NA;uncultured prymnesiophyte C19847|
                           Eukaryota;NA;NA;Pavlovales;|
                           Eukaryota;NA;NA;Phaeocystales;|
                           Eukaryota;NA;NA;Prymnesiales;|
                           Eukaryota;NA;NA;Syracosphaerales;",
                           superkingdom_phylum_class_order_family_genus_SPECIES))
Haptophyta$Phylum <- "Haptophyta"
```

```
Ochrophyta <- filter(Hist2F3Fdry, grepl("Eukaryota;Bacillariophyta;|Eukaryota;Bolidophyceae;|
                            Eukaryota;Eustigmatophyceae;|
                            Eukaryota;NA;Chrysomerophyceae;|
                            Eukaryota;NA;Chrysophyceae;|
                            Eukaryota;NA;Dictyochophyceae;|
                            Eukaryota;NA;NA;NA;NA;Olisthodiscus;|
                            Eukaryota;NA;NA;NA;NA;Phalansterium;|
                            Eukaryota;NA;NA;NA;NA;Schizocladia;|
                            Eukaryota;NA;Pelagophyceae;|
                            Eukaryota;NA;Phaeothamniophyceae;|
                            Eukaryota;NA;Raphidophyceae;|
                            Eukaryota;NA;Synchromophyceae;|
                            Eukaryota;NA;Synurophyceae;|
                            Eukaryota;Phaeophyceae;|Eukaryota;Pinguiophyceae;|
                            Eukaryota;Xanthophyceae;",
                            superkingdom_phylum_class_order_family_genus_SPECIES))
Ochrophyta$Phylum <- "Ochrophyta"
#Rhodophyta already determined above; just bind it to list of all algae
Rhodophyta$Phylum <- "Rhodophyta"
##now build up Algae dataframe
Algae <- filter(Hist2F3Fdry, grepl("Eukaryota;Chlorophyta|Eukaryota;Chromerida|
                         Eukaryota;Picozoa|Eukaryota;Streptophyta",
                         superkingdom_phylum_class_order_family_genus_SPECIES))
Algae <- bind_rows(Algae, Cercozoa, Cryptista, Dinozoa, Euglenozoa, Glaucophyta,
Haptophyta,
                Ochrophyta, Rhodophyta)

##Construct dataframes for plotting
#replace all NA entries for phylum/class/order
#with "incertae sedis" (uncertain phylogenetic placement)
##BACTERIA
#Bacteria WITH Cyanos, for stacked bar graphs
TwoFBacterialSpeciesPhylum <- BacteriaWithCyanos %>% select(Species, Phylum, Mean2F,
sd2F) %>%
                                    rename(Mean=Mean2F, sd=sd2F) %>%
                                    arrange(-Mean) %>%
                                    mutate(Phylum=replace(Phylum, Phylum=="NA",
                                                "*incertae sedis"))
#Bacteria WITHOUT Cyanos, top 20 for bar graphs
TwoFBacterialSpeciesNoCyanosPhylum20 <- BacteriaNoCyanos %>%
                                select(Species, Phylum, Mean2F, sd2F) %>%
                                rename(Mean=Mean2F, sd=sd2F) %>% arrange(-Mean) %>%
                                mutate(Phylum=replace(Phylum, Phylum=="NA",
                                            "*incertae sedis")) %>%
                                top_n(20, Mean)
```

```
ThreeFBacterialSpeciesPhylum <- BacteriaWithCyanos %>% select(Species, Phylum, Mean3F,
sd3F) %>%
                                    rename(Mean=Mean3F, sd=sd3F) %>%
                                    arrange(-Mean) %>%
                                    mutate(Phylum=replace(Phylum, Phylum=="NA",
                                                "*incertae sedis"))
ThreeFBacterialSpeciesNoCyanosPhylum20 <- BacteriaNoCyanos %>%
                                select(Species, Phylum, Mean3F, sd3F) %>%
                                rename(Mean=Mean3F, sd=sd3F) %>% arrange(-Mean) %>%
                                mutate(Phylum=replace(Phylum, Phylum=="NA",
                                            "*incertae sedis")) %>%
                                top_n(20, Mean)
##section below is data specific and should be run interactively
A <- distinct(TwoFBacterialSpeciesNoCyanosPhylum20, Phylum); A
B <- distinct(ThreeFBacterialSpeciesNoCyanosPhylum20, Phylum); B
bacphy <- union(A, B)
#add phylum to 2F dataset at position row = 21 to have same phylum key
TwoFBacterialSpeciesNoCyanosPhylum20 <- TwoFBacterialSpeciesNoCyanosPhylum20 %>%
                            add_row(Species="", Phylum="(Gemmatimonadetes)",
                                Mean=0, sd=0)

##ARCHAEA
TwoFArchaealSpeciesPhylum <- Archaea %>% select(Species, Phylum, Mean2F, sd2F) %>%
                             rename(Mean=Mean2F, sd=sd2F) %>% arrange(-Mean) %>%
                             mutate(Phylum=replace(Phylum, Phylum=="NA",
                                        "*incertae sedis"))
TwoFArchaealSpeciesPhylum20 <- TwoFArchaealSpeciesPhylum %>% top_n(20, Mean)
ThreeFArchaealSpeciesPhylum <- Archaea %>% select(Species, Phylum, Mean3F, sd3F) %>%
                              rename(Mean=Mean3F, sd=sd3F) %>% arrange(-Mean) %>%
                              mutate(Phylum=replace(Phylum, Phylum=="NA",
                                         "*incertae sedis"))
ThreeFArchaealSpeciesPhylum20 <-ThreeFArchaealSpeciesPhylum %>% top_n(20, Mean)
##section below is data specific and should be run interactively
A <- distinct(TwoFArchaealSpeciesPhylum20, Phylum); A
B <- distinct(ThreeFArchaealSpeciesPhylum20, Phylum); B
#okay as is

##FUNGI
TwoFFungalSpeciesPhylum <- Fungi %>% select(Species, Phylum, Mean2F, sd2F) %>%
                           rename(Mean=Mean2F, sd=sd2F) %>%
                           arrange(-Mean) %>%
                           mutate(Phylum=replace(Phylum, Phylum=="NA",
                                      "*incertae sedis"))
TwoFFungalSpeciesPhylum20 <- TwoFFungalSpeciesPhylum  %>% top_n(20, Mean)
ThreeFFungalSpeciesPhylum <- Fungi %>% select(Species, Phylum, Mean3F, sd3F) %>%
                            rename(Mean=Mean3F, sd=sd3F) %>%
```

```
                        arrange(-Mean) %>%
                        mutate(Phylum=replace(Phylum, Phylum=="NA",
                                "*incertae sedis"))
ThreeFFungalSpeciesPhylum20 <- ThreeFFungalSpeciesPhylum %>% top_n(20, Mean)
##section below is data specific and should be run interactively
A <- distinct(TwoFFungalSpeciesPhylum20, Phylum); A
B <- distinct(ThreeFFungalSpeciesPhylum20, Phylum); B
funphy <- union(A, B); funphy
# add phylum to 2F dataset at position row = 21 to have same phylum key
TwoFFungalSpeciesPhylum20 <- TwoFFungalSpeciesPhylum20 %>%
                  add_row(Species="", Phylum="(*incertae sedis)", Mean=0, sd=0) %>%
                  add_row(Species="", Phylum="(Mucoromycota)", Mean=0, sd=0)

##CYANOBACTERIA
TwoFCyanoSpeciesOrder <- Cyanobacteria %>% select(Species, Order, Mean2F, sd2F) %>%
                rename(Mean=Mean2F, sd=sd2F) %>%arrange(-Mean) %>%
                mutate(Order=replace(Order, Order=="NA", "*incertae sedis"))
TwoFCyanoSpeciesOrder20 <- TwoFCyanoSpeciesOrder %>% top_n(20, Mean)
ThreeFCyanoSpeciesOrder <- Cyanobacteria %>% select(Species, Order, Mean3F, sd3F) %>%
                rename(Mean=Mean3F, sd=sd3F) %>% arrange(-Mean) %>%
                mutate(Order=replace(Order, Order=="NA", "*incertae sedis"))
ThreeFCyanoSpeciesOrder20 <- ThreeFCyanoSpeciesOrder %>% top_n(20, Mean)
##section below is data specific and should be run interactively
A <- distinct(TwoFCyanoSpeciesOrder20, Order); A
B <- distinct(ThreeFCyanoSpeciesOrder20, Order); B
cyaphy <- union(A, B); cyaphy
#add order to 2F dataset at position row = 21 to have same order key
TwoFCyanoSpeciesOrder20 <- TwoFCyanoSpeciesOrder20 %>%
                  add_row(Species="", Order="(Chroococcidiopsidales)", Mean=0, sd=0)
ThreeFCyanoSpeciesOrder20 <- ThreeFCyanoSpeciesOrder20 %>%
                  add_row(Species="", Order="(Gloeobacterales)", Mean=0, sd=0)

##CHLOROPHYTES
TwoFChlorophyteSpeciesClass <- Chlorophyta %>% select(Species, Class, Mean2F, sd2F)
%>%
                        rename(Mean=Mean2F, sd=sd2F) %>%
                        arrange(-Mean) %>%
                        mutate(Class=replace(Class, Class=="NA",
                                "*incertae sedis"))
TwoFChlorophyteSpeciesClass20 <- TwoFChlorophyteSpeciesClass %>% top_n(20, Mean)
ThreeFChlorophyteSpeciesClass <- Chlorophyta %>% select(Species, Class, Mean3F, sd3F)
%>%
                        rename(Mean=Mean3F, sd=sd3F) %>%
                        arrange(-Mean) %>%
                        mutate(Class=replace(Class, Class=="NA",
                                "*incertae sedis"))
```

```
ThreeFChlorophyteSpeciesClass20<- ThreeFChlorophyteSpeciesClass %>% top_n(20, Mean)
##section below is data specific and should be run interactively
A <- distinct(TwoFChlorophyteSpeciesClass20, Class); A
B <- distinct(ThreeFChlorophyteSpeciesClass20, Class); B
#okay as is

##RHODOPHYTES
TwoFRhodophyteSpeciesClass <- Rhodophyta %>% select(Species, Class, Mean2F, sd2F) %>%
                    rename(Mean=Mean2F, sd=sd2F) %>% arrange(-Mean) %>%
                    mutate(Class=replace(Class, Class=="NA", "*incertae sedis"))
TwoFRhodophyteSpeciesClass20 <- TwoFRhodophyteSpeciesClass %>% top_n(20, Mean)
ThreeFRhodophyteSpeciesClass <- Rhodophyta %>% select(Species, Class, Mean3F, sd3F)
%>%
                     rename(Mean=Mean3F, sd=sd3F) %>% arrange(-Mean) %>%
                     mutate(Class=replace(Class, Class=="NA", "*incertae sedis"))
ThreeFRhodophyteSpeciesClass20<- ThreeFRhodophyteSpeciesClass %>% top_n(20, Mean)
##section below is data specific and should be run interactively
A <- distinct(TwoFRhodophyteSpeciesClass20, Class); A
B <- distinct(ThreeFRhodophyteSpeciesClass20, Class); B
#okay as is

#most of below is not used, just used for Stacked Bar Chart of Fig. 1
TwoFAlgaeSpeciesPhylum <- Algae %>% select(Species, Phylum, Mean2F, sd2F) %>%
                          rename(Mean=Mean2F, sd=sd2F) %>% arrange(-Mean) %>%
                          mutate(Phylum=replace(Phylum, Phylum=="NA",
                                   "*incertae sedis"))
TwoFAlgaeSpeciesPhylum20 <- TwoFAlgaeSpeciesPhylum %>% top_n(20, Mean)
ThreeFAlgaeSpeciesPhylum <- Algae %>% select(Species, Phylum, Mean3F, sd3F) %>%
                           rename(Mean=Mean3F, sd=sd3F) %>% arrange(-Mean) %>%
                           mutate(Phylum=replace(Phylum, Phylum=="NA",
                                    "*incertae sedis"))
ThreeFAlgaeSpeciesPhylum20<- ThreeFAlgaeSpeciesPhylum %>% top_n(20, Mean)


####################################
### FIGURE 1: Stacked Bar Charts ###
####################################
##PANELS A & B
##Bacterial Phyla Stacked Bar Chart
A <- TwoFBacterialSpeciesPhylum %>% group_by(Phylum) %>%
summarize(rawsum=sum(Mean),
                                        sd=sqrt(sum(sd^2))) %>%
                       mutate(Type="2F") %>% mutate(TotalSum=sum(rawsum),
                                    Total_sd=sqrt(sum(sd^2)),
                                    Proportion=rawsum/TotalSum*100) %>%
                       select(Phylum, Type, Proportion, TotalSum, Total_sd)
```

```
B <- ThreeFBacterialSpeciesPhylum %>% group_by(Phylum) %>%
summarize(rawsum=sum(Mean),
                                        sd=sqrt(sum(sd^2))) %>%
                          mutate(Type="3F") %>% mutate(TotalSum=sum(rawsum),
                                                       Total_sd=sqrt(sum(sd^2)),
                                                     Proportion=rawsum/TotalSum*100) %>%
                          select(Phylum, Type, Proportion, TotalSum, Total_sd)
#top 10 phyla only
bacterialphyla <- bind_rows(A %>% arrange(-Proportion) %>% top_n(10, Proportion), B %>%
                      arrange(-Proportion) %>% top_n(10, Proportion)); bacterialphyla
#write out table
write_tsv(bacterialphyla, path="Dry Bacterial Phyla Proportions.tsv")
#HACK to get same width plots with LONG names in Archaeal Phyla and short names in Fungal
Phyla
bacterialphyla <- bacterialphyla %>% mutate(Phylum=replace(Phylum,
Phylum=="Deinococcus-Thermus",
                            "Deinococcus-Thermus               ")) # 16 extra characters
#reorder factors so biggest is on bottom; make a stacked bar chart of percentages
bacterialphyla$Phylum = with(bacterialphyla, reorder(Phylum, +Proportion, mean))
#make a stacked bar chart of percentages from A and B above
p1=ggplot(bacterialphyla) + aes(fill=Phylum, y=Proportion, x=Type) +
  geom_bar(position="fill", stat="identity") +
  annotate("text", x=1, y=1.05, label= "(41.1±1.6)\n%", size=2) +
  annotate("text", x=2, y=1.05, label= "(38.0±2.1)\n%", size=2) +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  scale_fill_viridis(discrete = T, direction = -1) + labs(tag = "A", fill='Phylum: Bacteria'); p1
##Archaea Phyla Stacked Bar Chart
A <- TwoFArchaealSpeciesPhylum %>% group_by(Phylum) %>%
summarize(rawsum=sum(Mean),
                                          sd=sqrt(sum(sd^2))) %>%
                        mutate(Type="2F") %>% mutate(TotalSum=sum(rawsum),
                                                     Total_sd = sqrt(sum(sd^2)),
                                                   Proportion=rawsum/TotalSum*100) %>%
                        select(Phylum, Type, Proportion, TotalSum, Total_sd)
B <- ThreeFArchaealSpeciesPhylum %>% group_by(Phylum) %>%
summarize(rawsum=sum(Mean),
                                          sd=sqrt(sum(sd^2))) %>%
                        mutate(Type="3F") %>% mutate(TotalSum=sum(rawsum),
                                                     Total_sd=sqrt(sum(sd^2)),
                                                   Proportion=rawsum/TotalSum*100) %>%
                        select(Phylum, Type, Proportion, TotalSum, Total_sd)
#top 10 phyla only
archaealphyla <- bind_rows(A %>% arrange(-Proportion) %>% top_n(10, Proportion),
                     B %>% arrange(-Proportion) %>% top_n(10, Proportion)); archaealphyla
```

```
#write out table
write_tsv(archaealphyla, path="Dry Archaeal Phyla Proportions.tsv")
#reorder factors so biggest is on bottom; make a stacked bar chart of percentages
archaealphyla$Phylum = with(archaealphyla, reorder(Phylum, +Proportion, mean))
#make a stacked bar chart of percentages from A and B above
p2=ggplot(archaealphyla, aes(fill=Phylum, y=Proportion, x=Type)) +
  geom_bar(position="fill", stat="identity") +
  annotate("text", x=1, y=1.05, label= "(8.0±0.9)\n/100 %", size=2) +
  annotate("text", x=2, y=1.05, label= "(8.8±0.2)\n/100 %", size=2) +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black")) +
  scale_fill_viridis(discrete = T, direction = -1) + labs(tag = "B", fill='Phylum: Archaea'); p2
##Plot array of graphs using patchwork
#export each plot in 4" x 8.5" landscape mode; Fig1X-Y....
(p1 + p2); ggsave(file="Fig1A-B.BacteriaArchaeaProportions-v2.pdf",
                  plot=last_plot(), scale=1, width=8.5, height=4, dpi=300, units=c("in"))

##PANELS C & D
##Fungal Phyla Stacked Bar Chart
A <- TwoFFungalSpeciesPhylum %>% group_by(Phylum) %>%
summarize(rawsum=sum(Mean),
                                          sd=sqrt(sum(sd^2))) %>%
                          mutate(Type="2F") %>% mutate(TotalSum = sum(rawsum),
                                                       Total_sd = sqrt(sum(sd^2)),
                                                      Proportion = rawsum/TotalSum*100) %>%
                          select(Phylum, Type, Proportion, TotalSum, Total_sd)
B <- ThreeFFungalSpeciesPhylum%>% group_by(Phylum) %>%
summarize(rawsum=sum(Mean),
                                            sd=sqrt(sum(sd^2))) %>%
                         mutate(Type="3F") %>% mutate(TotalSum = sum(rawsum),
                                                      Total_sd = sqrt(sum(sd^2)),
                                                     Proportion = rawsum/TotalSum*100) %>%
                         select(Phylum, Type, Proportion, TotalSum, Total_sd)
#top 10 phyla only
fungalphyla <- bind_rows(A %>% arrange(-Proportion) %>% top_n(10, Proportion),
                         B %>% arrange(-Proportion) %>% top_n(10, Proportion)); fungalphyla
#write out table
write_tsv(fungalphyla, path="Dry Fungal Phyla Proportions.tsv")
#HACK to get same width plots with LONG names in Archaeal Phyla and short names in Fungal
Phyla
fungalphyla <- fungalphyla %>% mutate(Phylum=replace(Phylum,
Phylum=="Neocallimastigomycota",
                          "Neocallimastigomycota               ")) # 16 extra characters
#reorder factors so biggest is on bottom; make a stacked bar chart of percentages
fungalphyla$Phylum = with(fungalphyla, reorder(Phylum, +Proportion, mean))
#make a stacked bar chart of percentages from A and B above
```

```
p3=ggplot(fungalphyla, aes(fill=Phylum, y=Proportion, x=Type)) +
  geom_bar(position="fill", stat="identity") +
  annotate("text", x=1, y=1.05, label= "(7.4±0.3)\n/10 %", size=2) +
  annotate("text", x=2, y=1.05, label= "(8.27±0.05)\n/10 %", size=2) +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  scale_fill_viridis(discrete = T, direction = -1) + labs(tag = "C", fill='Phylum: Fungi'); p3
##All Algae Phyla Stacked Bar Chart
A <- TwoFAlgaeSpeciesPhylum %>% group_by(Phylum) %>%
summarize(rawsum=sum(Mean),
                                        sd=sqrt(sum(sd^2))) %>%
                        mutate(Type="2F") %>% mutate(TotalSum = sum(rawsum),
                                        Total_sd = sqrt(sum(sd^2)),
                                       Proportion = rawsum/TotalSum*100) %>%
                        select(Phylum, Type, Proportion, TotalSum, Total_sd)
B <- ThreeFAlgaeSpeciesPhylum%>% group_by(Phylum) %>%
summarize(rawsum=sum(Mean),
                                        sd=sqrt(sum(sd^2))) %>%
                       mutate(Type="3F") %>% mutate(TotalSum = sum(rawsum),
                                        Total_sd = sqrt(sum(sd^2)),
                                       Proportion = rawsum/TotalSum*100) %>%
                       select(Phylum, Type, Proportion, TotalSum, Total_sd)
#top 10 phyla only
algalphyla <- bind_rows(A %>% arrange(-Proportion) %>% top_n(10, Proportion),
                    B %>% arrange(-Proportion) %>% top_n(10, Proportion)); algalphyla
#write out table
write_tsv(algalphyla, path="Dry Algal Phyla Proportions.tsv")
#HACK to get same width plots with LONG names in Archaeal Phyla and short names in Fungal
Phyla
algalphyla <- algalphyla %>% mutate(Phylum=replace(Phylum, Phylum=="Chlorophyta",
                        "Chlorophyta                              ")) # 31 extra characters
#reorder factors so biggest is on bottom; make a stacked bar chart of percentages
algalphyla$Phylum = with(algalphyla, reorder(Phylum, +Proportion, mean))
#make a stacked bar chart of percentages from A and B above
p4=ggplot(algalphyla, aes(fill=Phylum, y=Proportion, x=Type)) +
  geom_bar(position="fill", stat="identity") +
  annotate("text", x=1, y=1.05, label= "(1.8±0.1)\n/10 %", size=2) +
  annotate("text", x=2, y=1.05, label= "(4.4±0.4)\n/10 %", size=2) +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black")) +
  scale_fill_viridis(discrete = T, direction = -1) + labs(tag = "D", fill='Phylum: "Algae"'); p4
#export PDF in 4" x 8.5" landscape
(p3 + p4); ggsave(file="Fig1C-D.FungiAlgaeProportions.pdf",
                plot=last_plot(), scale=1, width=8.5, height=4, dpi=300, units=c("in"))
```

```
##PANELS E & F
##Chloropyte Classes Stacked Bar Chart
A <- TwoFChlorophyteSpeciesClass %>% group_by(Class) %>%
summarize(rawsum=sum(Mean),
                                        sd=sqrt(sum(sd^2))) %>%
                          mutate(Type="2F") %>% mutate(TotalSum=sum(rawsum),
                                        Total_sd=sqrt(sum(sd^2)),
                                      Proportion=rawsum/TotalSum*100) %>%
                          select(Class, Type, Proportion, TotalSum, Total_sd)
B <- ThreeFChlorophyteSpeciesClass %>% group_by(Class) %>%
summarize(rawsum=sum(Mean),
                                         sd=sqrt(sum(sd^2))) %>%
                           mutate(Type="3F") %>% mutate(TotalSum=sum(rawsum),
                                         Total_sd=sqrt(sum(sd^2)),
                                       Proportion=rawsum/TotalSum*100) %>%
                           select(Class, Type, Proportion, TotalSum, Total_sd)
#top 10 phyla only
chlorophyteclass <- bind_rows(A %>% arrange(-Proportion) %>% top_n(10, Proportion),
                      B %>% arrange(-Proportion) %>% top_n(10, Proportion))
                      chlorophyteclass
#write out table
write_tsv(chlorophyteclass, path="Dry Chlorophyte Classes Proportions.tsv")
#HACK to get same width plots with LONG names in Archaeal Phyla and short names in Fungal
Phyla
chlorophyteclass <- chlorophyteclass %>% mutate(Class=replace(Class,
                                  Class=="Nephroselmidophyceae",
                            "Nephroselmidophyceae              ")) # 15 extra characters
#reorder factors so biggest is on bottom; make a stacked bar chart of percentages
chlorophyteclass$Class = with(chlorophyteclass, reorder(Class, +Proportion, mean))
#make a stacked bar chart of percentages from A and B above
p5=ggplot(chlorophyteclass, aes(fill=Class, y=Proportion, x=Type)) +
  geom_bar(position="fill", stat="identity") +
  annotate("text", x=1, y=1.05, label= "(0.74±0.08)\n/10 %", size=2) +
  annotate("text", x=2, y=1.05, label= "(1.8±0.2)\n/10 %", size=2) +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black")) +
  scale_fill_viridis(discrete = T, direction = -1) + labs(tag = "E",
                                       fill='Class: Chlorophytes'); p5
##Rhodophyte Classes Stacked Bar Chart
A <- TwoFRhodophyteSpeciesClass %>% group_by(Class) %>%
summarize(rawsum=sum(Mean),
                                         sd=sqrt(sum(sd^2))) %>%
                          mutate(Type="2F") %>% mutate(TotalSum = sum(rawsum),
                                         Total_sd=sqrt(sum(sd^2)),
                                       Proportion = rawsum/TotalSum*100) %>%
                          select(Class, Type, Proportion, TotalSum, Total_sd)
```

```
B <- ThreeFRhodophyteSpeciesClass %>% group_by(Class) %>%
summarize(rawsum=sum(Mean),
                                        sd=sqrt(sum(sd^2))) %>%
                          mutate(Type="3F") %>% mutate(TotalSum=sum(rawsum),
                                        Total_sd=sqrt(sum(sd^2)),
                                      Proportion=rawsum/TotalSum*100) %>%
                          select(Class, Type, Proportion, TotalSum, Total_sd)
#top 10 phyla only
rhodophyteclass <- bind_rows(A %>% arrange(-Proportion) %>% top_n(10, Proportion),
                        B %>% arrange(-Proportion) %>% top_n(10, Proportion))
                        rhodophyteclass
#write out table
write_tsv(rhodophyteclass, path="Dry Rhodophyte Classes Proportions.tsv")
#HACK to get same width plots with LONG names in Archaeal Phyla and short names in Fungal
Phyla
rhodophyteclass <- rhodophyteclass %>% mutate(Class=replace(Class,
Class=="Compsopogonophyceae",
                              "Compsopogonophyceae          ")) # 12 extra characters
#reorder factors so biggest is on bottom; make a stacked bar chart of percentages
rhodophyteclass$Class = with(rhodophyteclass, reorder(Class, +Proportion, mean))
#make a stacked bar chart of percentages from A and B above
p6=ggplot(rhodophyteclass, aes(fill=Class, y=Proportion, x=Type)) +
  geom_bar(position="fill", stat="identity") +
  annotate("text", x=1, y=1.05, label= "(0.44±0.09)\n/10 %", size=2) +
  annotate("text", x=2, y=1.05, label= "(1.5±0.3)\n/10 %", size=2) +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        panel.background = element_blank(), axis.line = element_line(colour = "black")) +
  scale_fill_viridis(discrete = T, direction = -1) + labs(tag = "F",
                                        fill='Class: Rhodophytes'); p6
#export PDF in 4" x 8.5" landscape
(p5 + p6); ggsave(file="Fig1E-F.ChlorophytesRhodophytesProportions.pdf",
              plot=last_plot(), scale=1, width=8.5, height=4, dpi=300, units=c("in"))


###############################################
### FIGURE 2: WHOLE & SPLIT PLOT ANALYSES ###
###############################################
##WHOLE PLOT ANALYSIS: cf. differences between 2F and 3F microbiomes
#make a grouping factor based on slothname; NOTE: ORDER of levels IS CRITICAL
slothname<-factor(TwoF3Fdry$Name, levels=c(unique(TwoF3Fdry$Name)))
#Levels: Cher Freddie Gwen Judy Madonna Aladdin Buzz Esperanza Merlin Shuri Tarzan
#generate a bray-curtis distance matrix from the reads per taxon dataframe with sloths on rows
readspertaxon.dry.noNA <- select(TwoF3Fdry, -label, -Index, -FileID, -Name, -Type, -Location,
                          -Season) %>% mutate_all(~replace(., is.na(.), 0.))
microbetaxondist.dry <- vegdist(readspertaxon.dry.noNA, method="bray") #, na.rm=TRUE)
#run betadisper()
```

```
betadispresults.dry <- betadisper(microbetaxondist.dry, slothname, type = "centroid")
#create an object of the centroids
centroids.wholeplot <- betadispresults.dry$centroids
#The resulting object contains values for each treatment and site combination for each PCO axis.
#obtain and attach a factor file with 4 observations,
#one for each treatment by site combination, between.subjects.factors
#create reduced factor dataframe from meta dataframe
#NOTE: order should match levels=c(unique(TwoF3Fdry$name)) above!
reducedfactors.dry <- as.data.frame(distinct(select(meta, name, type)))
# name   type
# Cher 2F
# Freddie        2F
# Gwen           2F
# Judy 2F
# Madonna      2F
# Aladdin        3F
# Buzz 3F
# Esperanza    3F
# Merlin         3F
# Shuri3F
# Tarzan         3F
#note that "type" is type of sloth, either 2F or 3F
perMOV.whole <- adonis(centroids.wholeplot~type, reducedfactors.dry, method = "euclidean")
perMOV.whole

## SPLIT PLOT ANALYSIS: cf. differences (between) location and type of sloth
fullfactors.dry <- as.data.frame(distinct(select(meta, name, type, location)))
perMOVsplit <- adonis(readspertaxon.dry.noNA~name+location+location:type, fullfactors.dry,
                      method = "bray")
perMOVsplit
#run ordination
mdsord.dry <- metaMDS(readspertaxon.dry.noNA, distance="bray")
#to obtain response scores for metaMDS, make a matrix from the sample scores#
nmdsscores <- as.data.frame(scores(mdsord.dry))
#add metadata to nmdsscores data frame for plotting
nmdsscores$name <- TwoF3Fdry$Name
nmdsscores$type <- TwoF3Fdry$Type
nmdsscores$location <- TwoF3Fdry$Location
nmdsscores <- nmdsscores %>% unite(Legend, type, location, sep="_", remove=FALSE)
#graph with ggplot with 95% confidence ellipses; export as 4" x 6" PDF
ggplot(nmdsscores, aes(x=NMDS1, y=NMDS2, colour=Legend, shape=Legend)) +
  stat_ellipse(size=0.75, show.legend = FALSE) + geom_point(size=3) + labs(fill="Legend") +
  scale_shape_manual(values=c(17,19,17,19)) + #scale_size_manual(values=c(3,3,3,3)) +
  theme(text = element_text(size = 14), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
```

```
  theme(legend.key=element_blank()) +
  scale_color_manual(values=c("#E69F00", "#F0E442", "#0072B2", "#56B4E9"))
#export NMDS plot in 5" x 7" landscape mode; Fig2....
ggsave(file="NMDS-plot-slothDrySeason-NormSampleReads-v2.pdf",
       plot=last_plot(), scale=1, width=7, height=5, dpi=300, units=c("in"))


############################################################
### FIGURE 3: Bar Charts for Dry Season 2F and 3F taxa ###
############################################################
##PANEL A: 2F Sloth Bacterial Species
library(gridExtra)
TwoFBacterialSpeciesNoCyanosPhylum20$Phylum =
with(TwoFBacterialSpeciesNoCyanosPhylum20,
                                    reorder(Phylum, -Mean, mean))
p1<-ggplot(data=TwoFBacterialSpeciesNoCyanosPhylum20, aes(x=reorder(Species, Mean),
y=Mean)) +
  geom_bar(stat="identity", color="black", aes(fill=Phylum), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % Sample Reads", x="", title="Two-Fingered Sloth") +
  scale_y_continuous(breaks = c(0, 1, 2, 3, 4)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(1,21.7), ylim=c(0,4.07), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "A", fill="Phylum: Bacteria") + theme(legend.position=c(.62, .3)); p1
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot1 <- p1 + theme(plot.title = element_text(hjust = -1)); plot1
##PANEL B: 3F Sloth Bacterial Species - Top 20 Phyla
ThreeFBacterialSpeciesNoCyanosPhylum20$Phylum =
with(ThreeFBacterialSpeciesNoCyanosPhylum20,
                                      reorder(Phylum, -Mean, mean))
p2<-ggplot(data=ThreeFBacterialSpeciesNoCyanosPhylum20, aes(x=reorder(Species, Mean),
y=Mean)) +
  geom_bar(stat="identity", color="black", aes(fill=Phylum), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % Sample Reads", x="", title = "Three-Fingered Sloths") +
  scale_y_continuous(breaks = c(0, 1, 2, 3, 4)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(0,20.7), ylim=c(0,4.07), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
```

```
  labs(tag = "B", fill="Phylum: Bacteria") + theme(legend.position=c(.65, .3)); p2
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot2 <- p2 + theme(plot.title = element_text(hjust = -2.75)); plot2
##Plot array of graphs using patchwork
##export each plot in 7" x 14" landscape mode; Fig3X-Y....
(plot1 + plot2)
ggsave(file="Fig3A-B.Bacteria.pdf",
       plot=last_plot(), scale=1, width=14, height=7, dpi=300, units=c("in"))

##PANEL C: 2F Sloth Archaeal Species
TwoFArchaealSpeciesPhylum20$Phylum = with(TwoFArchaealSpeciesPhylum20,
reorder(Phylum, -Mean,
                                                    mean))
p3<-ggplot(data=TwoFArchaealSpeciesPhylum20, aes(x=reorder(Species, Mean), y=Mean)) +
  geom_bar(stat="identity", color="black", aes(fill=Phylum), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % Sample Reads", x = "", title = "Two-Fingered Sloths") +
  scale_y_continuous(breaks = c(0, 0.002, 0.004, 0.005)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(0,20.7), ylim=c(0,0.0043), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "C", fill="Phylum: Archaea") + theme(legend.position=c(.65, .25))
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot3 <- p3 + theme(plot.title = element_text(hjust = -13)); plot3
##PANEL D: 3F Sloth Archaeal Species
ThreeFArchaealSpeciesPhylum20$Phylum = with(ThreeFArchaealSpeciesPhylum20,
reorder(Phylum, -Mean,
                                                      mean))
p4<-ggplot(data=ThreeFArchaealSpeciesPhylum20, aes(x=reorder(Species, Mean), y=Mean)) +
  geom_bar(stat="identity", color="black", aes(fill=Phylum), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % Sample Reads", x = "", title = "Three-Fingered Sloths") +
  scale_y_continuous(breaks = c(0, 0.002, 0.004, 0.005)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(0,20.7), ylim=c(0,0.0043), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "D", fill="Phylum: Archaea") + theme(legend.position=c(.65, .25))
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot4 <- p4 + theme(plot.title = element_text(hjust = 24)); plot4
##export each plot in 7" x 14" landscape mode; Fig3X-Y....
```

```
(plot3 + plot4)
ggsave(file="Fig3C-D.Archaea.pdf",
      plot=last_plot(), scale=1, width=14, height=7, dpi=300, units=c("in"))

##PANEL E: 2F Sloth Fungal Species
TwoFFungalSpeciesPhylum20$Phylum = with(TwoFFungalSpeciesPhylum20, reorder(Phylum,
-Mean, mean))
p5<-ggplot(data=TwoFFungalSpeciesPhylum20, aes(x=reorder(Species, Mean), y=Mean)) +
  geom_bar(stat="identity", color="black", aes(fill=Phylum), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % of Sample Reads", x = "", title = "Two-Fingered Sloths") +
  scale_y_continuous(breaks = c(0, 0.1, 0.2)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(1,21.7), ylim=c(0,0.21), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "E", fill="Phylum: Fungi") + theme(legend.position=c(.65, .25)); p5
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot5 <- p5 + theme(plot.title = element_text(hjust = -0.25)); plot5
##PANEL F: 3F Sloth Fungal Species
ThreeFFungalSpeciesPhylum20$Phylum = with(ThreeFFungalSpeciesPhylum20,
reorder(Phylum, -Mean,
                                                    mean))
p6<-ggplot(data=ThreeFFungalSpeciesPhylum20, aes(x=reorder(Species, Mean), y=Mean)) +
  geom_bar(stat="identity", color="black", aes(fill=Phylum), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % of Sample Reads", x = "", title = "Three-Fingered Sloths") +
  scale_y_continuous(breaks = c(0, 0.1, 0.2)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(0,20.7), ylim=c(0,0.21), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "F", fill="Phylum: Fungi") + theme(legend.position=c(.65, .25)); p6
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot6 <- p6 + theme(plot.title = element_text(hjust = -0.4)); plot6
# export each plot in 7" x 14" landscape mode; Fig3X-Y....
(plot5 + plot6)
ggsave(file="Fig3E-F.Fungi.pdf",
      plot=last_plot(), scale=1, width=14, height=7, dpi=300, units=c("in"))

##PANEL G: 2F Sloth Cyanobacterial Species
```

```
TwoFCyanoSpeciesOrder20$Order = with(TwoFCyanoSpeciesOrder20, reorder(Order, -Mean,
mean))
#shorten: Cyanobacteria bacterium 13_1_40CM_2_61_4 ==> Cyanobacterium
13_1_40CM_2_61_4
#        & Cyanobacteria bacterium 13_1_20CM_4_61_6 ==> Cyanobacterium
13_1_20CM_4_61_6
TwoFCyanoSpeciesOrder20 <- TwoFCyanoSpeciesOrder20 %>%
mutate(Species=replace(Species,
                       Species=="Cyanobacteria bacterium 13_1_40CM_2_61_4",
                             "Cyanobacterium 13_1_40CM_2_61_4")) %>%
                       mutate(Species=replace(Species,
                                              Species=="Cyanobacteria bacterium 13_1_20CM_4_61_6",
                                              "Cyanobacterium 13_1_20CM_4_61_6"))
p7<-ggplot(data=TwoFCyanoSpeciesOrder20, aes(x=reorder(Species, Mean), y=Mean)) +
  geom_bar(stat="identity", color="black", aes(fill=Order), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % of Sample Reads", x = "", title = "Two-Fingered Sloths") +
  scale_y_continuous(limits = c(0, 0.073), breaks = c(0, 0.02, 0.04, 0.06, 0.08)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(1,21.7), ylim=c(0,0.073), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "G", fill="Order: Cyanobacteria") + theme(legend.position=c(.62, .25)); p7
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot7 <- p7 + theme(plot.title = element_text(hjust = -1.1)); plot7

##PANEL H: 3F Sloth Cyanobacterial Species
#note that error bars for these are all very large so don't show
ThreeFCyanoSpeciesOrder20$Order = with(ThreeFCyanoSpeciesOrder20, reorder(Order, -
Mean, mean))
p8<-ggplot(data=ThreeFCyanoSpeciesOrder20, aes(x=reorder(Species, Mean), y=Mean)) +
  geom_bar(stat="identity", color="black", aes(fill=Order), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % of Sample Reads", x = "", title = "Three-Fingered Sloths") +
  scale_y_continuous(limits = c(0, 0.069), breaks = c(0, 0.02, 0.04, 0.06, 0.08)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(1,21.7), ylim=c(0,0.073), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "H", fill="Order: Cyanobacteria") + theme(legend.position=c(.71, .21)); p8
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot8 <- p8 + theme(plot.title = element_text(hjust = -1.3))
```

```
##export each plot in 7" x 14" landscape mode; Fig3X-Y....
(plot7 + plot8)
ggsave(file="Fig3G-H.Cyanobacteria.pdf",
      plot=last_plot(), scale=1, width=14, height=7, dpi=300, units=c("in"))

##PANEL I: 2F Sloth Chlorophyte Species
TwoFChlorophyteSpeciesClass20$Class = with(TwoFChlorophyteSpeciesClass20,
reorder(Class, -Mean,
                                                    mean))
p9<-ggplot(data=TwoFChlorophyteSpeciesClass20, aes(x=reorder(Species, Mean), y=Mean)) +
#  geom_bar(stat="identity", color="dark green", aes(fill=Class), width=0.5) +
  geom_bar(stat="identity", color="black", aes(fill=Class), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % of Sample Reads", x = "", title = "Two-Fingered Sloths") +
  scale_y_continuous(breaks = c(0, 0.005, 0.01, 0.015, 0.02)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(0,20.7), ylim=c(0,0.0157), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "I", fill="Class: Chlorophytes") + theme(legend.position=c(.7, .15)); p9
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot9 <- p9 + theme(plot.title = element_text(hjust = -0.7)); plot9
##PANEL J: 3F Sloth Chlorophyte Species
ThreeFChlorophyteSpeciesClass20$Class = with(ThreeFChlorophyteSpeciesClass20,
                                  reorder(Class, -Mean, mean))
p10<-ggplot(data=ThreeFChlorophyteSpeciesClass20, aes(x=reorder(Species, Mean), y=Mean))
+
#  geom_bar(stat="identity", color="dark green", aes(fill=Class), width=0.5) +
  geom_bar(stat="identity", color="black", aes(fill=Class), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % of Sample Reads", x = "", title = "Three-Fingered Sloths") +
  scale_y_continuous(breaks = c(0, 0.005, 0.01, 0.015, 0.02)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(0,20.7), ylim=c(0,0.0157), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "J", fill="Class: Chlorophytes") + theme(legend.position=c(.7, .15)); p10
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot10 <- p10 + theme(plot.title = element_text(hjust = -1)); plot10
##export each plot in 7" x 14" landscape mode; Fig3X-Y....
(plot9 + plot10)
ggsave(file="Fig3I-J.Chlorophytes.pdf",
```

```
      plot=last_plot(), scale=1, width=14, height=7, dpi=300, units=c("in"))

##PANEL K: 2F Sloth Rhodophyte Species
TwoFRhodophyteSpeciesClass20$Class = with(TwoFRhodophyteSpeciesClass20, reorder(Class,
-Mean,
                                                    mean))
p11<-ggplot(data=TwoFRhodophyteSpeciesClass20, aes(x=reorder(Species, Mean), y=Mean)) +
#  geom_bar(stat="identity", color="red3", aes(fill=Class), width=0.5) +
  geom_bar(stat="identity", color="black", aes(fill=Class), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % of Sample Reads", x = "", title = "Two-Fingered Sloths") +
  scale_y_continuous(breaks = c(0, 0.015, 0.03)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(0,20.7), ylim=c(0,0.035), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "K", fill="Class: Rhodophytes") + theme(legend.position=c(.65, .25)); p11
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot11 <- p11 + theme(plot.title = element_text(hjust = -0.25)); plot11
##PANEL L: 3F Sloth Rhodophyte Species
ThreeFRhodophyteSpeciesClass20$Class = with(ThreeFRhodophyteSpeciesClass20,
                                            reorder(Class, -Mean, mean))
p12<-ggplot(data=ThreeFRhodophyteSpeciesClass20, aes(x=reorder(Species, Mean), y=Mean))
+
#  geom_bar(stat="identity", color="red", aes(fill=Class), width=0.5) +
  geom_bar(stat="identity", color="black", aes(fill=Class), width=0.5) +
  geom_errorbar(aes(ymin=Mean-sd, ymax=Mean+sd), width=.1, position=position_dodge(.9)) +
  theme(text = element_text(size = 16), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), panel.background = element_blank(),
        axis.line = element_line(colour = "black")) +
  labs(y= "Average % of Sample Reads", x = "", title = "Three-Fingered Sloths") +
  scale_y_continuous(breaks = c(0, 0.015, 0.03)) +
  scale_fill_viridis(discrete = T, direction = -1) +
  coord_flip(xlim=c(0,20.7), ylim=c(0,0.035), expand=FALSE) +
  theme(axis.text = element_text(face = "italic", color = "black")) +
  labs(tag = "L", fill="Class: Rhodophytes") + theme(legend.position=c(.65, .25)); p12
#hack the centering of the title...can't seem to do automatically in Patchwork...
plot12 <- p12 + theme(plot.title = element_text(hjust = -0.6)); plot12
##export each plot in 7" x 14" landscape mode; Fig3X-Y....
(plot11 + plot12)
ggsave(file="Fig3K-L.Rhodophytes.pdf",
      plot=last_plot(), scale=1, width=14, height=7, dpi=300, units=c("in"))
```

```
#############################################################
### DIVERSITY INDICES ANALYSIS AND PLOTS for TABLE 4 ###
#############################################################
##readspertaxon.dry.noNA from NMDS section above, uses TwoF3Fdry dataframe
diversityscores <- select(TwoF3Fdry, label, Type)
diversityscores$H <- diversity(readspertaxon.dry.noNA)
diversityscores$H
diversityscores$Simpson <- diversity(readspertaxon.dry.noNA, "simpson")
diversityscores$Simpson
diversityscores$InverseSimpson <- diversity(readspertaxon.dry.noNA, "inv")
diversityscores$InverseSimpson
diversityscores$Shannon <- diversity(readspertaxon.dry.noNA, index = "shannon",
                            MARGIN = 1, base = exp(1));
diversityscores$Shannon
write_tsv(diversityscores, "DiversityIndices-Dry2F3FSpecies.tsv")
##calculate summary stats using Rmisc::summarySE function
#note, Rmisc messes up/redefines prior commands! so load here and last...
library(Rmisc)
Simpsonsummary<- summarySE(diversityscores, measurevar="Simpson", groupvars=c("Type"))
Simpsonsummary
InvSimpsonsummary<- summarySE(diversityscores, measurevar="InverseSimpson",
groupvars=c("Type"));
InvSimpsonsummary
Shannonsummary<- summarySE(diversityscores, measurevar="Shannon", groupvars=c("Type"))
Shannonsummary
##write out results
write_tsv(Simpsonsummary, "DiversityIndicesSummary-Dry2F3FSpecies.tsv")
write_tsv(InvSimpsonsummary, "DiversityIndicesSummary-Dry2F3FSpecies.tsv",
        append=TRUE, col_names=TRUE)
write_tsv(Shannonsummary, "DiversityIndicesSummary-Dry2F3FSpecies.tsv", append=TRUE,
col_names=TRUE)
```

VITA

EDUCATION

Bachelor of Arts, Biology, Willamette University, 2017
Advisor: Dr. David Craig

PRESENTATIONS

Kaup, M., Hom, E.Y., *Elucidating the Sloth Hair Microbiome: A Metagenomic Comparison of Two- and Three-fingered Sloths*. American Society of Microbiology- South Central Branch, Oxford MS. November 2019.

Kaup, M. *The Secrets of Sloths and Their Symbionts*. Sequoia Park Zoo Conservation Lecture Series, Eureka CA. December 2018.

Kaup, M. *A Taste of What We Waste*. TEDxSalem. Salem OR. November 2015.

EMPLOYMENT

University of Mississippi, *Teaching Assistant*	2017 – 2020
Environmental Protection Agency, *Greater Research Opportunities Fellowship*	2015 – 2017
Willamette University, *Writing Center Consultant*	2014 – 2016
Willamette University, *Teaching Assistant*	2015
Willamette University, *Tutor*	2014 – 2015
Willamette University, *Green Initiative Fund Intern*	2014

HONORS AND AWARDS

University of Mississippi Who's Who Class of 2018/2019	2019
Phi Beta Kappa Inductee	2017