ABSTRACT

THOEMMES, MEGAN STROUP. Mites, Microbes, and the History of Mammalian Species Interactions. (Under the direction of Dr. Robert R. Dunn).

Species interactions are dictated by both host genetics and the environment, and these associations can be reflective of their shared evolutionary past. For example, the microscopic species that live on mammal bodies can carry a genetic signature of host diversification and movement through time. This is particularly true for the species that are closely associated or that have a long history co-diversification. For mammals, one such group is *Demodex* mites. *Demodex* are microscopic, live below the skin's surface, and (due to their size and habitat niche) would likely require close contact to move among individuals. In addition, unique *Demodex* species have been found on all mammals tested to date, suggesting these mites are likely to have co-diversified with their hosts. Here, we quantified the prevalence and genetic diversity of human-associated *Demodex* (*D. folliculorum* and *D. brevis*). We found evidence that these mites are ubiquitously found on all adults, and their pattern of global genetic diversity is reflective of the divergence of ancient human populations.

However, mites are not the only organisms that have evolved alongside humans. There are trillions of microbes on the body, and our immune systems have developed in the context of the species that we come into contact with throughout our lives. This is important to consider, as we have moved into urbanized areas and attempted to seal ourselves off from the outdoors. Changes in our architecture and lifestyle have affected the diversity and types of species on us and around us, in ways that are detrimental to human health. And though it has been suggested that our interactions have changed over time, we don't fully understand what those associations were historically. Here, we used chimpanzee beds in Tanzania and pastoralist homes in Namibia as referential models to consider what our interactions might have been as we transitioned from the open nightly structures built by ancient humans, to more permanent homes, and then to

contemporary houses. We found that as structures became more permanent and sealed from the outdoors, there was less accumulation of diverse environmental bacteria (e.g., soil associates known to aid in immune development). In turn, we saw an increase in the accumulation of bacteria sourced from the skin, mouth, and feces. Overall, it appears that our biggest transition, in terms of species interactions, occurred when we began to settle into more permanent homes and remove ourselves from the outdoor environment.

Finally, we applied what we learned from human homes to the structures of other mammals. Human-built, supplemental nests are often used to increase available habitat and provide protection from predators and the environment for threatened and endangered species. Though supplemental nests model the function of their natural counterparts, they are commonly built from manufactured materials and incompletely mimic the design of natural nests. Based on our previous research, we know that architectural design is a strong determinant of the diversity and types of microbes found on interior surfaces. A loss in microbial diversity or the accumulation of pathogens in supplemental nests could have detrimental effects, particularly for species at a high risk of extinction. Here, we compared the bacterial communities found in natural and supplemental nests of the endangered Key Largo woodrat (*Neotoma floridana smalli*). Although we found distinct bacterial communities in Key Largo woodrat nests, there was no difference between natural and supplemental structures. Therefore, our data suggest that supplemental nests are not altering microbial species interactions in the ways we might have predicted, despite their differences in building material and design.

Mites, Microbes, and the History of Mammalian Species Interactions

by
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DEDICATION

To my son, E. Hemi Thoemmes, for being my light. May you never lose your sense of love and curiosity for the natural world. To my parents, John and Paula Stroup, for showing me what it means to be dedicated, work hard, and move through life with gratitude and generosity. And to all of my friends and family, for it is your continual love and support that holds me together and makes my life richer and more fulfilled.

BIOGRAPHY

Every particular in nature, a leaf, a drop, a crystal, a moment of time is related to the whole, and partakes of the perfection of the whole.

– Ralph Waldo Emerson

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Ubiquity and Diversity of Human-Associated *Demodex* Mites

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Abstract

Demodex mites are a group of hair follicle and sebaceous gland-dwelling species. The species of these mites found on humans are arguably the animals with which we have the most intimate interactions. Yet, their prevalence and diversity have been poorly explored. Here we use a new molecular method to assess the occurrence of *Demodex* mites on humans. In addition, we use the 18S rRNA gene (18S rDNA) to assess the genetic diversity and evolutionary history of *Demodex* lineages. Within our samples, 100% of people over 18 years of age appear to host at least one *Demodex* species, suggesting that *Demodex* mites may be universal associates of adult humans. A phylogenetic analysis of 18S rDNA reveals intraspecific structure within one of the two named human-associated *Demodex* species, *D. brevis*. The *D. brevis* clade is geographically structured, suggesting that new lineages are likely to be discovered as humans from additional geographic regions are sampled.

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Introduction

Many organisms live on us and in us. Fewer than 10% of the cells in our bodies are actually our own. Most of these organisms are bacteria, but we are also colonized by multicellular species including fungi [1], intestinal worms [2] and ectoparasites, such as lice [3–5], with nearly 2000 pathogen and parasite species alone known from human bodies [6]. Among the more enigmatic of the multicellular species that live on humans, as well as on other mammals, are mites of the genus *Demodex* (reviewed in [7]), which are common on human faces and other parts of the body [8,9]. While these mites are well known to dermatologists, ophthalmologists, and veterinarians and have been the subject of study for 172 years (reviewed in [10]), their ubiquity, diversity and evolution are poorly understood. For example, *Demodex* have not been sampled from the vast majority of mammal species, including those that seem very likely to host *Demodex* mites, such as chimpanzees and gorillas. Nor have most human populations been sampled for these mites.

Two species of *Demodex, D. brevis* (Akbulatova 1963) and *D. folliculorum* (Simon 1842), have been described from the human body. In general, *Demodex* live mostly within hair follicles. Biopsies of skin cross-sections reveal *D. folliculorum* to inhabit the area of the follicle above the sebaceous gland, where they appear to ingest cell contents [11]. *D. brevis*, on the other hand, primarily inhabits the sebaceous glands associated with vellus hairs [11], typically at densities of just one to a few mites per gland. With approximately 5 million hair follicles spread across the body [12] and more than 7 billion humans on Earth, the total habitat area available to these mites is immense. Methods used to collect *Demodex* mites from humans include biopsy, the cellophane tape method (placing tape on the face to stick to the mites), scraping areas where mites are likely to reside, and plucking eyelash and eyebrow hairs. Based on the visual observation of mites collected from healthy individuals by these methods, it appears that approximately 3–55% of humans harbor *Demodex*, with most studies falling in the range of 10–20% [8,13–16]. However, because these mites may occur in patches around the body, as in dogs [17], and all existing collection methods sample just small patches of skin (and even incompletely sample those patches), it is difficult to know to what extent the absence of mites in a sample equates to the absence of mites on the body. Intriguingly, in postmortem studies, mites appear to be present on all adult cadavers (reviewed in [10]). The ubiquity of mites on cadavers might indicate they are universally present on living, adult humans but missed by current sampling methods. Alternately, conditions in which cadavers are found might facilitate colonization by mites and, in doing so, artificially inflate estimates of their incidence.

Even less well understood than the proportion of people (or for that matter, other mammals) that host *Demodex* mites is the diversity of those mites. While two species of human-associated mites have been formally named, they were named based on morphological characters alone [18,19]. Given that *Demodex* mites inhabit restrictive, specialized environments (hair follicles), some aspects of their morphology, including their small size (~100–200 μM) and general elongate appearance, could reflect convergent evolution among distinct lineages or species groups which would only be discerned by examination of non-morphological data, e.g. by DNA sequence-based differences. A recent study of

human *Demodex* species found genetic differences in the mitochondrial CO1 gene between mite populations that inhabit the eyelashes versus mite populations that inhabit the skin [20]. In addition, studies of another human-associated parasite, lice (*Pediculus humanus*), have found strong genetic structure between geographic lineages [4,5,21]. Geographic structure among human-associated *Demodex* lineages is expected, given that these mites are more intimately associated with the body than lice and seemingly less mobile, yet the minimal data that exist have not yet recovered such variation [22]. Conversely, if *Demodex* lack strong geographic structure, it suggests the movement of mites among humans must occur very frequently (perhaps even with social greeting rituals) and across large geographic distances.

Only recently have molecular studies begun to consider *Demodex* mites. Existing phylogenies and estimates of molecular divergence include very limited sampling of *Demodex* species, are based on few genetic markers, and include only minimal geographic representation. The DNA sequences that have been obtained from human-associated *Demodex* species come almost exclusively from China (*D. folliculorum* and *D. brevis*) and Spain (*D. folliculorum)* [20,22]. Studies based on the 16S rRNA gene (16S rDNA) find little variation within *D. folliculorum* and show no geographic structure between samples from China and Spain [22]. However, no molecular data have been considered from *D. brevis* outside of China, and low genetic variation observed for human-associated *Demodex* in previous phylogenies [22] may reflect insufficient sampling rather than the actual genetic diversity of *Demodex* mites.

Here we test a new molecular approach to detect the presence of mites on human bodies and assess the proportion of individuals in one population colonized by mites. We then use phylogenetic reconstruction based on the nuclear 18S rRNA gene (18S rDNA) to better understand the diversity of these mites.

Materials and Methods

Ethics Statement

Participants were sampled by project staff at outreach events. Prior to sampling, each participant was verbally informed about the goals of the project and the sampling protocol. All participants were provided and signed a written Informed Consent form. All human *Demodex* sampling procedures and the participant Informed Consent form were approved by North Carolina State University's Institutional Review Board for the Protection of Human Subjects in Research (IRB), Approval No. 2966.

(a) Sample collection

All sample collections were performed in Raleigh, NC at either the North Carolina Museum of Natural Sciences or North Carolina State University. Each participant was gently scraped with a metal laboratory spatula along the creases of the nose and over the surrounding cheek area. The facial habitats were chosen based on their high levels of sebum production and ease of pore expression. In addition, Bonnar *et al*. (1993) found the greatest abundance of mites in the cheek area among rosacea patients [23]. Mineral oil was typically applied to the sampled area to facilitate mite removal. After collection, the sebum was moved to a drop of mineral oil on a cover slip fragment where it was inspected to note the presence or absence of visually identifiable mites within the sample. Regardless of the presence or absence of observed mites the entire cover slip fragment with the sebum and mineral oil was transferred to a 1.5 ml microcentrifuge tube and maintained in −20°C for subsequent DNA extraction.

(b) DNA Extraction and PCR

DNA was extracted from the sebum of individual participants, regardless of the presence or absence of an observed mite, using a Qiagen DNeasy Blood & Tissue kit. We followed the manufacturer's supplementary insect protocol, without the initial grinding step. The samples were incubated overnight at 56°C with 180 µl of ATL buffer and 20 µl proteinase K. The final elution step was performed with 150 µl of elution buffer warmed to 56°C.

We used either OneTaq (NEB) or TaKaRa Ex Taq (Clontech), which possess proofreading functions, for all PCR reactions to reduce polymerase induced sequence errors. We designed the primers by aligning all available *Demodex* 16S rDNA or 18S rDNA sequences across the same genes from several other mites and from humans. In an attempt to design primers that were likely to be unbiased with regards to *Demodex* and have a low affinity for the hosts' DNA, we selected priming sites near the 5′ and 3′ ends of most available *Demodex* sequences that were highly conserved among these mites, yet that were unlikely to amplify these genes from humans. The 16S rDNA primer sequences used were 5′-GGTATTTTGACTGTGCTAAGG-3′ and 5′-AAAARCCAA-CATCGAGGTA-3′, which amplify the region from nucleotide 26 to 358 of *D. folliculorum* sequence FN424245.1. The PCR cycling conditions for 16S rDNA were 94°C for 1 min, followed by 40 cycles of 94°C for 20 s, 47°C for 30 s, 72°C for 1 min and a final 72°C extension for 5 min. The 18S rDNA primers were 5′-GTTGAKCCTGCCAGTAGTCA-3′ and 5′-GTCTGAA-GACCTCACTAAATC-3′, which amplify the region from nucleotide 7 to 1688 of *D. folliculorum* sequence JF784006.1. The PCR cycling conditions for 18S rDNA were 94°C for 1 min, followed by 40 cycles of 94°C for 20 s, 45°C for 30 s, 72°C for 2 min and a final 72°C extension for 5 min.

The 16S rDNA PCR products were separated on 2% agarose gels to assess presence or absence of mite DNA within a sample. Non-specific amplification of human 16S rDNA occasionally occurred but was easily discernible as an approximately 100 bp larger product (see Figure 1B, lane 4). For this analysis, a set of 19 individuals over 18 years of age and a second set of ten individuals 18 years of age were used. Several 16S rDNA PCR reactions were also sequenced to verify the specificity of the primers. However, data from this gene was not sequenced for most individuals, because this sequence was rather short (~325 bp) and did not contain many phylogenetically informative sites (i.e., two phylogenetically informative sites exist among our 16S rDNA sequences and the *D. folliculorum* sequences available on GenBank).

The 18S rDNA PCR products were sequenced from four individuals and used for phylogenetic analyses. We chose 18S rDNA for these analyses as this PCR works well with very little incident of non-specific bands (see Figure 1A). Furthermore, the transfer of mtDNA between closely related species has been frequently observed [24–26]. By using the nuclear 18S rDNA, we hope to decrease the likelihood of introgression obscuring population or species variation. All sequences were submitted to GenBank (Table 1).

(c) Sequencing and Phylogenetic Analysis

Because our faces have the potential to harbor many thousands of individual *Demodex* mites, we expect remnants of these mites to be present in our pores and on the surface of our faces, making the clean isolation of *Demodex* DNA from a single mite difficult. Thus, we presume that each of our scrapings is likely to harbor DNA from multiple mites. To obtain sequences from single copies of 18S rDNA from individual mites, we cloned the 18S rDNA PCR products using TOPO TA Cloning Kits (Invitrogen). We picked and sequenced a minimum of five colonies from each person

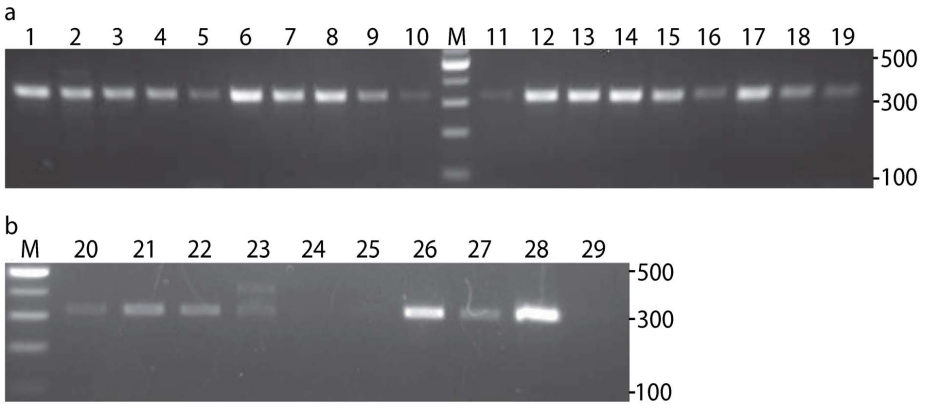

Figure 1. PCR based screen for presence of *Demodex* 16S rDNA in samples with no visually identifiable mites. Lanes labeled 1–29 represent samples from single individual participants. Lanes labeled M represent 100 bp molecular weight size markers. (a) PCR products indicate the presence of *Demodex* DNA in 100% of the screened samples from individuals over the age of 18. (b) PCR products indicate the presence of *Demodex* DNA in 70% of the screened samples from individuals 18 years of age.
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sampled in this study to get a sense of the diversity within an individual host. The resulting sequences were aligned with *Demodex* sequences available on GenBank using MAFFT v7 [27], with the E-INS-i algorithm, and checked by eye for best alignment. All GenBank sequences are named according to the species names given in GenBank; however, due to the current state of *Demodex* systematics some sequences are likely improperly designated (particularly dog-hosted species), leading to paraphyly of some taxa. The 18S rDNA sequence from a mite species, *Neochelacheles messersmithi*, in the same superfamily as *Demodex*, Cheyletoidea, was included as an outgroup for phylogenetic analysis.

To obtain estimates of genetic divergence between 18S rDNA sequences of all taxa included for phylogenetic analysis, Kimura 2-parameter distances (K2P) [28] and total number of nucleotide differences were calculated using MEGA v5 [29]. Genetic distances were calculated for all pairwise sequence comparisons as well as intra- and interspecific means.

Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI). Under both methods, gaps in the alignment were treated as missing data. jModelTest 2 [30] was used to determine the best-fitting model for the 18S rDNA data set. Using the corrected Akaike information criterion [31], the TIM2+ I + G model (with two rates of transitions and two rates of transversions) was selected as the best-fitting model for these data [32]. ML analysis was conducted using GARLI 2.0 for Windows [33]. Ten independent search replicates were run under the TIM2+ I + G model, with each replicate run for 100,000 generations. Bootstrap support values for nodes on the ML topology were computed with GARLI by running 1000 bootstrap replicates. The Bayesian analysis was conducted with MrBayes 3.2 [34]. Two independent runs were performed for 50 million generations, each with four chains (three heated and one cold), uninformative priors, and trees sampled at intervals of 1000 generations. Stationarity was determined by examining standard deviation of split frequencies between the two runs for convergence and examination of average potential scale reduction factor (PSRF). Of the 50,000 trees sampled in each run, the first 10,000 trees were discarded as burn-in and the remaining trees were used to construct a 50% majority rule consensus tree. Because the standard deviation of split frequencies was observed to drop and remain below 0.01 by 1,500,000 generations (i.e., 1500 sampled trees), our burn-in value of 10,000 was chosen to ensure that trees were sampled well after runs had reached convergence. The harmonic mean of likelihoods was estimated for post burn-in trees using the *sump* command in MrBayes. We assigned putative species sources for new sequences based solely on phylogenetic distance of previously reported species.

Results

Based on the observation of visually identifiable mite specimens within our samples, the prevalence of mites in adults was 14% (n = 253), in line with previous studies [8,13–16]. However, we were able to extract *Demodex* 16S rDNA from 100% of adults over the age of 18 (Figure 1A; Mean age: 37±10.4 years, n = 19). Molecular evidence suggests *Demodex* prevalence is much higher than recognized through visual observation alone. Our results are in line with postmortem studies that find *Demodex* mites present on all adult cadavers (reviewed in [10]).

Based on the observation of intact specimens in samples of young adults 18 years of age, mites were found on only 5.88% (n = 51). Of the ten 18 year olds we examined further for *Demodex* 16S rDNA, we amplified 16S rDNA PCR products from only seven samples (Figure 1B). Thus while 100% of adults in our sample hosted *Demodex* mite 16S rDNA, the prevalence and/or detectability in younger individuals appears lower (70%).

For phylogenetic analyses, we amplified, cloned, and sequenced *Demodex* 18S rDNA from four individual humans from whom we identified 17 unique *Demodex* 18S rDNA sequences (Table 1). These sequences reflect the presence of multiple mites within a given sample, even if we assume the presence of sequencing error and potential variation among 18S rDNA copies within the genome. We combined these sequences with previously published *Demodex* 18S rDNA sequences, representing at least 5 species from 4 mammalian hosts (human: *D. brevis* and *D. folliculorum*, dog: *D. canis*, mouse: *D. musculi*, and white-tailed deer: *D.* sp.) and an additional mite outgroup, *Neochelacheles messersmithi*, from the same superfamily as *Demodex*, Chelyetoidea (Figure 2). Our alignment comprised 1664 bp for 35 sequences (see Material

Table 1. *Demodex* mite species identification based on 18S rDNA gene sequence.

Host ID	Putative Species	Accession #	Length	Host Sex	Residence
127	*D. folliculorum*	KF745876	1636	M	USA
127	*D. brevis*	KF745877	1645	M	USA
127	*D. brevis*	KF745878	1646	M	USA
127	*D. brevis*	KF745879	1007*	M	USA
127	*D. brevis*	KF745880	1636	M	USA
141	*D. folliculorum*	KF745881	1636	F	USA
141	*D. brevis*	KF745882	1646	F	USA
141	*D. brevis*	KF745883	1643	F	USA
141	*D. brevis*	KF745884	1011*	F	USA
141	*D. brevis*	KF745885	1497*	F	USA
176	*D. folliculorum*	KF745886	1636	F	Brazil
176	*D. folliculorum*	KF745887	1636	F	Brazil
176	*D. folliculorum*	KF745888	1636	F	Brazil
315	*D. folliculorum*	KF745889	1636	F	Brazil
315	*D. folliculorum*	KF745890	1636	F	Brazil
315	*D. brevis*	KF745891	1646	F	Brazil
315	*D. brevis*	KF745892	1646	F	Brazil

The putative species assignment, GenBank accession number, and sequence length (bp) for each 18S rDNA gene sequence is listed, along with the ID, sex, and country of residence of the hosts. *Indicates partial sequences for which high-quality sequence data was not available for a portion of an amplified fragment.

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S1 for alignment). The ML analysis yielded a tree with the best score of –ln = 4887.29 (see Material S2 for ML tree file). The Bayesian analysis yielded a 50% consensus tree with harmonic mean of likelihood = −4976.76 (see Material S3 for Bayesian tree file). The average standard deviation of split frequencies of sampled trees = 0.00119, and the PSRF of sampled trees = 1.000. Phylogenetic analyses conducted with ML and BI yielded largely congruent topologies; minor incongruencies were restricted to placement of sequences with extremely short internodal branch lengths within the *D. folliculorum* clade and as such do not influence our interpretation. The ML topology is shown in Figure 2, with Bayesian posterior probabilities and ML bootstrap support values depicted adjacent to the major nodes of interest.

As evident in our phylogenetic results, we found substantial genetic diversity among (up to 0.065 K2P distance, up to 20 nucleotide substitutions (nts)) and within *Demodex* species (up to 0.032 K2P, up to 10 nts) (Table S1). Several of our sequences fit within a relatively well-supported *D. folliculorum* clade within which we find low genetic diversity (0.002 K2P, up to 2 nts) even though the individuals sampled included humans from North and South America and sequences from GenBank for individuals from China. Greater diversity is present within the *D. brevis* clade (up to 6.5 K2P, up to 10 nts). Multiple lineages of *D. brevis* appear to be present even on individual humans (within participant diversity: 0.006–0.007 K2P, 2–2.16 nts). However, the greatest diversity was among geographically distinct human populations (up to 0.032 K2P distance between American and Chinese sequences, 10 nts). Existing sequences of *D. brevis* sampled from humans in China resolve as a monophyletic clade sister to a New World clade composed of samples acquired for this study.

Discussion

Here we tested 29 people for the presence of *Demodex* mites and found that mites were much more common than expected in comparison to methods that rely solely on the visual confirmation of whole mite specimens taken from living humans. When we sampled individuals using traditional approaches, our results were similar to those of the many previous morphologically based studies [8,13–16]; 14% of individuals over the age of 18 had visually observed mites. But when we identified the presence of mites based on the amplification of *Demodex* DNA, we found that every adult over 18 years of age and 70% of 18 year olds had detectable *Demodex* 16S rDNA in the collected sebum of facial samples. Though it is possible *Demodex* 16S rDNA could be found on the face of an individual without mites, the likelihood that we detect such transferred DNA in our limited sampling area would be low. Moreover, if intact *Demodex* 16S rDNA were present in the environment at high enough levels to produce the results we see in adults, we would expect to see the same results among the 18 year olds, which we do not.

Little is known about the transmission of mites among humans. Recent studies find that many symbiotic microbes are passed directly from mother to offspring during breast-feeding [35] or during birth (especially if birth is vaginal) [36,37], and dogs acquire their *Demodex* mites as nursing pups [38]. In light of this, the same means of mite transmission seems possible in humans, supported by the fact that in one study, *Demodex* mites were found in 77% of nipple tissue from mastectomies [39]. Yet that we found mites on all adults but only 70% of 18 year olds, suggests that perhaps mite colonization does not strictly occur vertically, from parent to child. These results are in line with earlier morphological (largely postmortem) studies in which mites were found to be more prevalent on adults than on children (reviewed in [10]). Mites could be more ubiquitous on children than noted in postmortem studies or herein but at levels or in locations that make the mites difficult to detect even with the use of molecular approaches. One study of *Demodex* mites on Tokelau islanders found that mites were present on a greater number of children than on adults [40]. These conflicting findings highlight our limited understanding of how and when mites move onto and among human bodies.

Overall, we found the genetic variation of 18S rDNA within the genus *Demodex* comparable (up to 0.065 K2P) to the level of variation found among other genera within Acari (0.00–0.056 K2P; Ticks: Ixodidae) [41] (Table S1). This diversity suggests *Demodex* is a relatively old genus and even that the divergence between the two named human-associated species, *D. brevis* and *D. folliculorum*, might be relatively ancient. Within *Demodex*, *D. folliculorum* and *D. brevis* exhibit contrasting levels of intraspecific genetic diversity. *D. folliculorum*, which can be found living superficially within pores, show very little variation in the 18S rDNA sequence data we generated (mean of 0.002 K2P, up to 2 nts).

In comparison to *D. folliculorum*, *D. brevis* exhibited higher genetic diversity, not only between mites from the Americas and those from China (up to 0.032 K2P, up to 10 nts) but also among mites collected from the same individual human (0.005–0.009 K2P, 1.6–4.0 nts). Sequences of 18S rDNA from different *D. brevis* samples taken from the same face (of participant 141, Figure 2) exhibited more genetic variation (0.006 K2P, 4 nts) than those of *D. folliculorum* taken from Chinese and North and South Americans (mean 0.002 K2P). The diversity of *D. brevis* 18S rDNA found on individual humans suggests that not only do all adult humans have *Demodex* mites but that colonization is likely to occur more than once.

The Chinese *D. brevis* samples in GenBank and our newly generated samples from the Americas each form monophyletic clades with a relatively deep divergence between them (mean 0.021 K2P, 6.5 nts). The distance between the two *D. brevis* clades suggests strong geographic isolation among populations of *D. brevis*. Based on sequence divergence, these two populations are as different as are many congeneric species and subspecies. The 18S rDNA variation found between these two geographic populations is similar, for example, to that found between subspecies of parasitic lice, the head louse and body louse (*Pediculus humanus capitis* and *Pediculus humanus humanus*) [5]. *D. brevis* can be found more deeply embedded in sebaceous glands below the skin surface, in comparison to *D. folliculorum* that lives more superficially in the hair follicles. These contrasting habitat preferences may lead to more frequent transmission of *D. folliculorum* than of *D. brevis*, thus resulting in greater reproductive isolation and geographic structure in populations. However, given our limited geographic sampling, we expect the *Demodex* topology to change as samples from other regions are integrated.

The evolutionary history of the two human-associated *Demodex* species is, at best, poorly understood. *D. folliculorum* was described by Simon in 1842, and as late as 1933, all human *Demodex* were regarded as one, albeit variable, species [42,43]. It was only in 1963 that *D. brevis* was distinguished from *D. folliculorum* and described as a separate, but closely related, species [18]. Yet de Rojas *et al*. (2012) have demonstrated that interpreting variation in the morphology of the two human-associated *Demodex* mite species is problematic, even when interpreted in light of molecular (16S rDNA) sequence data [20]. The closest relatives for both human-associated species, *D. folliculorum* and *D. brevis,* remain unknown and are likely to remain unknown until these mites are much better sampled from other primates and mammalian hosts in general. Of the described

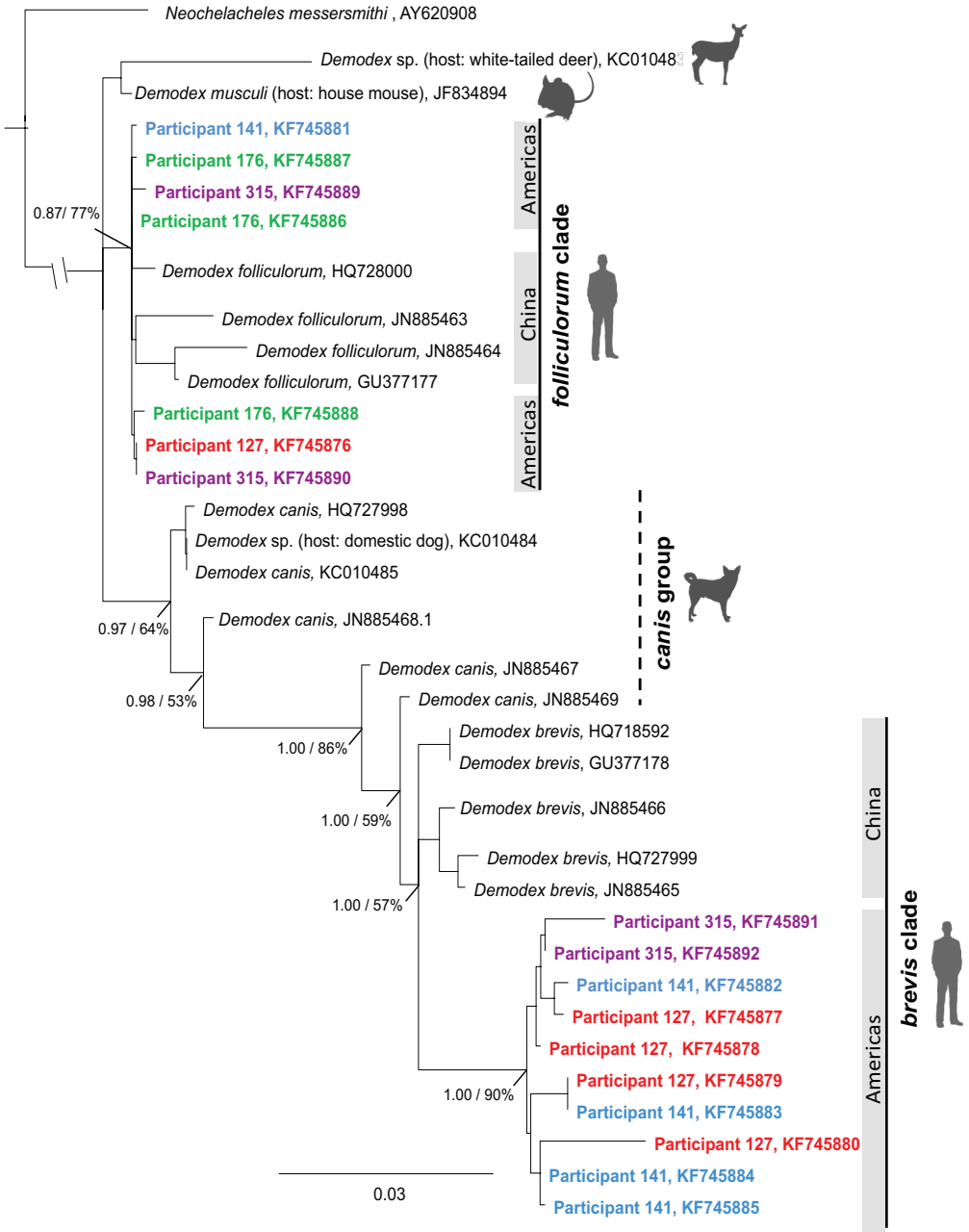

Figure 2. Maximum likelihood (ML) phylogeny of mites based on 18S rDNA sequences. Support values (Bayesian posterior probabilities/ML bootstrap support) given next to major nodes in the topology. Scale bar indicates the number of substitutions per site. Icons indicate mite host.
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Demodex species, only 13 have been sampled for molecular data and included in phylogenetic analyses. In addition, given that there are over 5000 species of mammals and as of yet, some mammals (such as humans, dogs, and cats) appear to host more than one *Demodex* species, any existing phylogeny represents a minute fraction of the possible species diversity of the genus.

Demodex are generally considered to be species specific, which would suggest there might be as many as 10,000 *Demodex* species on living mammals if there are two host specific mites per mammal species. Obviously, this estimate depends both on the ubiquity of *Demodex* mites among mammal species and on their true host specificity, both of which are poorly known.

Our phylogeny indicates that the two human-associated mite lineages do not share a recent common ancestor and likely have separate evolutionary histories of transmission to humans. The 18S rDNA sequence does not resolve the sister group to *D. folliculorum*, but places a paraphyletic group of dog-associated mites as the closest relative to *D. brevis*. The dog mite sequences included here were all acquired from GenBank and are primarily labeled *D. canis*. Yet, there are 3 morphologically distinct *Demodex* species that have been described from dogs (*D. canis*, *D. injai*, and *D. cornei*) and the molecular delimitation of these dog-associated species is not clear [44]. It seems likely that the sequences labeled *D. canis* included here may actually represent multiple dog-hosted *Demodex* species. Phylogenetic estimates based on 16S rDNA also find that dog-hosted *Demodex* mites share a recent common ancestor with a human-associated species, though in this case *D. folliculorum* and *D. brevis* are both more closely related to goat-associated mites, *D. caprae* [45]. The known habitat of *D. canis* is deep within the pores and is most similar to that of *D. brevis*. It is tempting to posit that *D. brevis* may have colonized humans from wolves during their domestication but any such assertion would be premature. Until other primate species are sampled, the mystery of whether humans acquired *Demodex* mites from our ape/hominid ancestors or through other means such as our interactions with domesticated mammal species will remain.

Supporting Information

Table S1 Pairwise distances between 18S rDNA sequences from *Demodex* species. Lower left = Kimura 2-parameter distances; Upper right = number of nucleotide differences.
(XLSX)

Material S1 *Demodex* 18S rDNA sequence alignment.
(FAS)

Material S2 Tree file for the maximum likelihood (ML) tree.
(TRE)

Material S3 Tree file for the Bayesian inference (BI) tree.
(TRE)

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Author Contributions

Conceived and designed the experiments: RRD MT MST DJF. Performed the experiments: MST DJF. Analyzed the data: DJF JU MT. Contributed reagents/materials/analysis tools: RRD JU. Wrote the paper: MST DJF JU MT RRD.

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CHAPTER 2: Ecology of Sleeping: The Microbial and Arthropod Associates of Chimpanzee Beds

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Ecology of sleeping: the microbial and arthropod associates of chimpanzee beds

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The indoor environment created by the construction of homes and other buildings is often considered to be uniquely different from other environments. It is composed of organisms that are less diverse than those of the outdoors and strongly sourced by, or dependent upon, human bodies. Yet, no one has ever compared the composition of species found in contemporary human homes to that of other structures built by mammals, including those of non-human primates. Here we consider the microbes and arthropods found in chimpanzee beds, relative to the surrounding environment ($n = 41$ and 15 beds, respectively). Based on the study of human homes, we hypothesized that the microbes found in chimpanzee beds would be less diverse than those on nearby branches and leaves and that their beds would be primarily composed of body-associated organisms. However, we found that differences

between wet and dry seasons and elevation above sea level explained nearly all of the observed variation in microbial diversity and community structure. While we can identify the presence of a chimpanzee based on the assemblage of bacteria, the dominant signal is that of environmental microbes. We found just four ectoparasitic arthropod specimens, none of which appears to be specialized on chimpanzees or their structures. These results suggest that the life to which chimpanzees are exposed while in their beds is predominately the same as that of the surrounding environment.

1. Introduction

Humans modify landforms and build complex networks of structures in which we gather in groups, store goods and protect ourselves from harsh environmental conditions. Since the advent of houses, which occurred between 20 000 [1–4] and 300 000 years ago [5], humans have become increasingly separated from the outdoor environment. Though there is cultural variation in the design and use of buildings globally, human interactions with other organisms now occur primarily within built structures [6]. It has been suggested that changes in the types and diversity of species with which we interact have been to our detriment, whether because we are no longer exposed to the diversity of environmental bacteria necessary for our immune systems to fully develop (e.g. the hygiene hypothesis [7]), or because we fail to acquire commensal species on which our physical health and mental well-being depend. A large body of the literature [8–13], including a number of recent high-profile books [14–17], now considers the idea that these shifts in our interactions with other organisms are making us sick. To varying extents, such work is predicated on the idea that our ancestors were exposed to more and different kinds of microbes than we are currently, whether through various daily activities or while they slept. Yet, to our knowledge no study has compared the species found in human homes, or more generally in the modern built environment, to those found in structures built by other mammals.

Many mammals sleep on the bare ground or in natural cavities, but a subset of mammals constructs modified structures in which to rest. The mammals that build these structures include rodents and other taxa that dig burrows [18–19] and a smaller group of mammals, including some primate species that build modified aboveground sleeping places referred to, variously, as roosts, nests or beds [20–21]. Great apes, including chimpanzees (*Pan troglodytes*), bonobos (*Pan paniscus*), gorillas (*Gorilla* spp.) and orangutans (*Pongo* spp.), all build at least one bed a day to be used for resting before abandonment the following morning [22]. Owing to the pervasiveness of this behaviour and the frequency of bed construction, it has been argued that these beds are the most prevalent form of technology and material culture among extant great apes [23–24]. Although great ape species differ in social organization, behaviour and diet, all construct their beds in a similar manner [22].

Chimpanzee beds, perhaps the best studied of the great ape beds, are complex structures built by interweaving branches into a secure foundation covered by a leafy mattress. These beds have been suggested to provide protection from the wind and other inclement weather, offer refuge from predators and increase comfort while resting. They are also hypothesized to reduce exposure to pests and pathogens [21,24–31]. Chimpanzees spend over half their lives in beds, and they are selective in the materials they use for construction, as well as to where they choose to build them [32–35]. Because chimpanzees spend many hours in their beds each day, these structures are likely to influence which species colonize the skin, guts and other habitats of chimpanzee bodies, and their exposures to such groups are likely to have an impact on their immune systems.

Here we consider the bacteria and arthropods found in chimpanzee beds. More specifically, we consider the diversity and likely origin of such species. Human homes are full of thousands of species that slough off our bodies or consume dead skin, food waste and the house materials themselves [36]. But it has been suggested that what is missing from many homes are the bacteria and other organisms associated with soils, leaves and outdoor habitats [7,8]. Implicitly, this body of research presumes that our ancestors were exposed to microbes and insects from diverse environmental sources, including during the hours in which they slept. We might predict the same for extant non-human great apes, such as chimpanzees. Alternatively, it may be that the overnight contact of chimpanzees with their beds is sufficient to allow body-associated organisms to accumulate, much as is the case for our own modern beds. To test these contrasting hypotheses, we sampled chimpanzee beds in the Issa Valley, western Tanzania.

2. Material and methods

The Issa valley is situated within the Greater Mahale Ecosystem in Tanzania. It is more than 90 km northeast from the nearest national park boundary (Mahale Mountains), and roughly 60 km southeast from the nearest town (Uvinza). This region is characterized by broad valleys, separated by steep mountains and flat plateaus, ranging from 900–1800 m above sea level. Vegetation is dominated by miombo woodland—*Brachystegia* and *Julbernardia* (Fabaceae), interspersed with swamp and grassland. A small proportion of the landscape (approximately 7%) is composed of evergreen gallery and thicket riverine forests. There are two distinct seasons: wet (November–April) and dry (May–October). Rainfall averages about 1200 mm per annum (range: 900–1400 mm, from 2001–2003; 2009–2014), and temperatures range from 11°C to 35°C [23,37]. The core study area (85 km^2) is used by one community of chimpanzees. As chimpanzees in Issa are unhabituated to observers, the exact number of individual builders represented is unknown; however, previous work by Rudicell *et al*. [38] estimated this community to include approximately 67 individuals.

Within the study area, we collected microbes from chimpanzee beds ($n = 41$) and from environmental locations ($n = 41$), as well as the arthropods associated with a subset of those beds ($n = 15$ beds and 15 forest floor locations). Samples were collected between August 2013 and April 2014. All chimpanzee beds were sampled following abandonment. Bed age was calculated as time since construction and grouped into one of three classes; fresh = 1 day, recent = 2–7 days and old = 11–35 days (following Plumptre & Reynolds, [39]). Because the beds in our study were not used for more than one night, time since abandonment and bed age are the same. Additionally, though we know the identity of the chimpanzee community, we could not directly observe which chimpanzee used a given bed; therefore, we do not consider how individual variation influences the bacteria and arthropods present. We focus instead on the overall differences in how organisms in chimpanzee beds vary relative to the natural habitat. Fieldwork was approved by the Tanzanian Wildlife Research Institute (TAWRI) and the Commission for Science and Technology (COSTECH); permit no. 2014-202-ER-2011-94.

2.1. Microbial collection, processing and analyses

Dust samples to be used in microbial analyses were collected using dual-tipped sterile BBL™ CultureSwabs™, identical to those used to study homes in the USA [36,40], as well as the International Space Station [41]. We collected dust from two sample locations within each chimpanzee bed; a branch used for bed construction ($n = 41$ beds) and, for a subset of beds, a leaf that composed the mattress ($n = 14$ beds). As branches provide the structural support for chimpanzee beds, we would expect frequent contact during building, general activity and rest. Additionally, we collected two environmental samples from within the same tree, at a height similar to that of the sampled bed; a branch not incorporated into the bed ($n = 41$ locations) and a leaf not incorporated into the mattress ($n = 14$ locations). These paired, environmental sites would have presumably had much less exposure time, if any at all, to the chimpanzees. For our analyses, we pooled branch and leaf samples and considered differences in surface type as a potential explanatory factor in determining microbial diversity and community composition.

For each sample, we performed DNA extractions with a MO BIO PowerSoil® DNA Isolation Kit (12888-100). Under sterile conditions, we removed one swab and swirled it against the side of a PowerBead tube for 10 s. We conducted all subsequent microbial DNA extraction steps in accordance with the provided kit protocol, apart from step 19, in which we reduced the quantity of Solution C6 to 50 µl to concentrate the eluted DNA. We then sent extracted DNA to the Microbiome Core Facility, University of North Carolina Chapel Hill, School of Medicine (USA) for PCR amplification and sequencing on the Illumina MiSeq platform. We targeted an approximately 300 bp sequence, within the V1–V2 region of the 16S rRNA gene, with universal primers: 8F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 338R 5′-GCTGCCTCCCGTAGGAGT-3′.

We merged overlapping reads with FLASH (v 1.2.11, [42]), set to allow a maximum overlap of 200 bp, and used the UPARSE pipeline (v 8.0.1623, [43]) to cluster sequences into operational taxonomic units (OTUs) at 97% similarity. We assigned taxonomy using the RDP Classifier 2.2 in QIIME [44,45], trained on the Greengenes database (v. 13_8, [46]), and identified a total of 8913 unique OTUs from 3 088 288 sequences. We removed low-quality or spurious OTUs by applying several filters to the dataset. OTUs were removed if they had a merged consensus sequence length outside the range of 310–370 bp, if they had less than 50 total reads across all samples, or if their taxonomy was flagged as cyanobacteria, mitochondria or unassigned (15% of total sequences; removed sequences in the electronic supplementary material, table S1). The filtered dataset contained 2 625 831 sequence reads over 1967 OTUs. We then

rarefied those sequences to 5600 reads per sample and used the rarefied dataset for all downstream analyses. Of our 96 samples, four samples from within chimpanzee beds and four environmental samples did not meet the minimum rarefaction threshold. We analysed all data in the R environment with the *mctoolsr* and *vegan* packages [47–49].

Using our rarefied dataset, we compared differences in OTU richness (measured by the number of unique OTUs within a sample) and the Shannon diversity index among samples with Kruskal–Wallis tests. We tested the relative contribution of each potential explanatory factor on both OTU richness and microbial community composition with permutational multivariate analysis of variance (PERMANOVA), based on 999 permutations [50]. We quantified differences among microbial communities through square-root transformation and the Bray–Curtis dissimilarity metric and visualized community composition data with nonmetric multidimensional scaling (NMDS) ordination plots. We included all potential explanatory variables of interest within both the OTU richness and community composition PERMANOVA models, using the false discovery rate correction for multiple comparisons. Variables within these models included whether a sample was from a chimpanzee bed, the age of a bed, season (wet or dry), elevation above sea level (metres), and whether a sample was from a branch or a leaf.

To assess the extent to which the microbial community within chimpanzee beds is dominated by taxa from the same sources as those that are most abundant in human beds (i.e. faecal, skin and oral associates; [36]), we used a source-tracking approach similar to those used previously [36,51]. While the microbiota of humans and chimpanzees differ, a number of bacterial taxonomic groups are characteristically associated with mammals [52,53], and an even larger number is shared among great apes [54–56]. In order to determine whether a bacterial taxon is likely to have come from the faeces, skin or mouth of a chimpanzee, it would be ideal to characterize the microbes from the wild chimpanzees within our study sites. However, since this population of chimpanzees is unhabituated, we used body associate data from previous research. We used data collected from wild and sanctuary primate populations within Africa to define a list of bacterial taxa associated with chimpanzee faeces and mouths (faecal: [57–59]; oral: [60]; electronic supplementary material, table S2). Where data from wild chimpanzees were not available (i.e. skin associates), we used taxonomic groups defined from the skin samples of captive chimpanzees [61] augmented with bacterial taxa found by Ross [52] to be ubiquitous across mammal orders, including those of non-human primates (electronic supplementary material, table S2). We do so while acknowledging that some taxa common on the skin of wild chimpanzees might be missing in captive populations (as seen in faeces; [62–63]) and absent from other mammals. However, given the similarity of skin microbiomes across mammal orders [52], we think this to be a reasonable starting point. We tested all differences in the relative abundance of body-associated microbes between bed and environmental samples with Kruskal–Wallis tests.

2.2. Arthropod collection and analyses

We collected arthropod specimens from 15 chimpanzee beds, at two locations per bed, using a handheld insect vacuum (BioQuip products); inside the bed and the ground directly below the bed ($n = 30$). We vacuumed each bed and ground location for 2 min. After collecting samples, we stored them in 95% ethanol and shipped them to R.R.D.'s laboratory (NC State University) for specimen sorting and identification. M.A.B. identified arthropods to the lowest possible taxonomic rank, based on morphology from intact specimens, in the NC State Entomology and Plant Pathology laboratory. Owing to the great diversity of poorly characterized invertebrate species in Tanzania, particularly in the canopy [64], we were unable to identify many of the specimens to species, or even family, level. However, because the arthropods associated with primates have been well studied [65], we were confident that we could identify such specimens if present.

We calculated arthropod richness based on the identification of morphospecies and tested differences in abundance between chimpanzee beds and the ground directly below each bed with a Poisson distribution. We also assessed the likelihood of arthropods in the samples being chimpanzee bed or human home associates and calculated the total number of known or potentially blood-feeding ectoparasites based on biological information provided in the literature for the taxa recovered [36,65]. Here we did not consider how arthropod communities vary with bed age. We found so few ectoparasites that it was impossible to formally analyse differences among bed and forest floor locations or to quantify changes over time, beyond reporting our raw counts and the identification of each of the collected specimens.

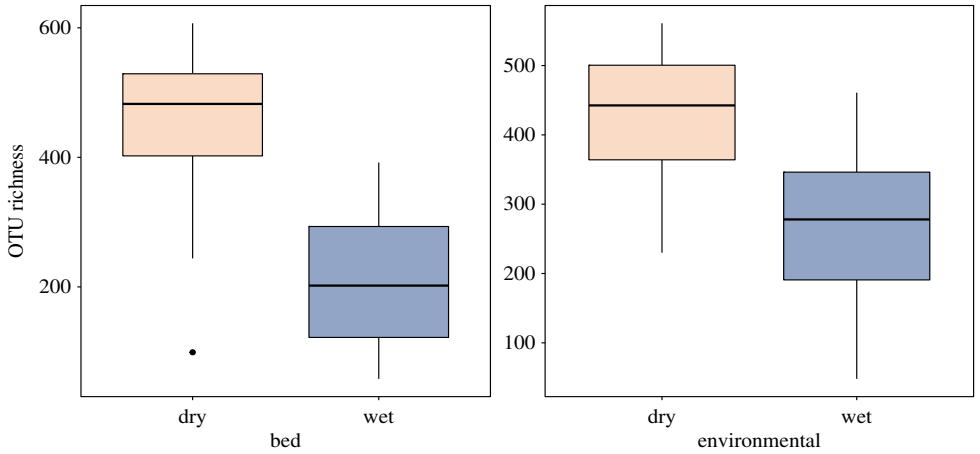

Figure 1. The OTU richness among all samples was primarily driven by differences in wet and dry seasons ($p < 0.001$). Season accounted for approximately 43% of the observed variation, with no difference between chimpanzee beds and the environment ($p = 0.509$). OTU richness was greatest in the dry season overall, as well as when chimpanzee beds or environmental samples were considered on their own ($R^2 = 0.54, p < 0.001$; $R^2 = 0.32, p < 0.001$, respectively).

3. Results

3.1. Microbes

We identified a total of 1896 microbial OTUs in chimpanzee beds and 1784 microbial OTUs from environmental samples. Proteobacteria, Actinobacteria and Bacteroidetes were the most common phyla, accounting for 92.4% of sequence reads from beds and 91.4% of sequence reads from environmental samples, with the phyla Proteobacteria and Actinobacteria accounting for nearly all OTUs present. The most common families of bacteria in both the chimpanzee beds and the surrounding environment were Methylocystaceae, Pseudonocardiaceae and Microbacteriaceae.

We observed no differences in the OTU richness or Shannon diversity of microbes in chimpanzee beds, when compared to branches and leaves of the same tree (richness: $\chi^2 = 0.071$, $p = 0.789$; average OTU richness per sample: bed = 343, tree branch or leaf = 357; Shannon diversity: $\chi^2 = 1.288$, $p = 0.256$). When considering the relative contribution of all factors, season was the strongest determinate of OTU richness across all samples. Whether samples were collected in the wet or dry season accounted for nearly half of the observed variation ($R^2 = 0.43$, $p < 0.001$), where richness was greatest during the dry season (figure 1). Elevation above sea level was the next most explanatory variable ($R^2 = 0.31$, $p = 0.011$). When considering only the microbes found in chimpanzee beds, age of the bed and whether samples were taken from branches or leaves did not affect OTU richness ($p = 0.631$, $p = 0.811$, respectively; electronic supplementary material, table S3*a*).

Just as with OTU richness, differences in community composition among all samples was strongly influenced by season ($p < 0.001$) and elevation above sea level ($p < 0.001$). However, here elevation explained 46% of the total observed variation, whereas season accounted for only 13% ($p < 0.001$). Within beds, the presence of one or more chimpanzees was a determinate of microbial community composition, though the effect was small relative to the other factors ($R^2 = 0.03$, $p < 0.001$; electronic supplementary material, figure S1). Bed age was not predictive of community assemblage ($p = 0.714$; electronic supplementary material, table S3*b*).

Of the top five most abundant bacterial genera known to be associated with chimpanzee faeces (as found in Yildirim *et al*. [58]), *Oscillabacter*, *Roseburia*, *Faecalibacterium* and *Caprococcus* were not found in any of our samples, regardless of whether the sample was collected in or outside of a chimpanzee bed. Even closely related genera in the *Oscillabacter* family, Oscillospiraceae, were not present. Faecal bacteria from the *Ruminococcus* genus were present but rare (occurred in just 5% of samples and accounted for 0.008% of sequence reads) and were no more abundant in beds than from environmental locations ($\chi^2 = 2.857$, $p = 0.090$). Even when we expanded our dataset to include all faecal taxa [57–59]; electronic supplementary material, table S2), we found no difference in the proportion of faecal bacteria present in

Table 1. Arthropod specimens. (Specimens were identified to the family or group level. Presence/absence data were noted for chimpanzee bed and ground samples. All specimens indicated as parasites are from taxa that include ectoparasites.)

class	order	family or group	nest	ground	notes
Arachnida	Sarcoptiformes	Oribatida	X	X	single specimen in nest
		Astigmata		X	
	Trombidiformes	Erythraeidae		X	
		Bdelloidea		X	
	Mesostigmata	unidentified		X	
	unidentified 'Acari'	unidentified		X	
	Araneae	Oonopidae	X		
		Oxyopidae		X	
		Salticidae		X	
		Selenopidae	X		
		unidentified	X	X	
	Pseudoscorpionida	unidentified		X	
Diplopoda	Polyxenida	unidentified	X	X	
Insecta	Collembola[a]	Entomobryidae	X	X	
		Symphypleona		X	
		unidentified	X	X	
	Zygentoma	Lepismatidae	X		
	Isoptera	unidentified		X	
	Orthoptera	Mogoplistidae	X	X	
		Tettigoniidae	X		
		unidentified	X	X	
	Blattodea	Ectobiidae		X	
	Hemiptera	Aphididae		X	
		Blissidae	X		
		Cicadellidae	X	X	
		Coccoidea	X		
		Dipsocoridae		X	
		Fulgoroidea	X	X	
		Lasiochilidae	X		
		Psylloidea	X	X	
		Reduviidae	X	X	
		Rhyparochromidae		X	
		Veliidae		X	*Hebrovelia*
		other Anthocoroidea[a]	X		
		other Auchenorrhyncha	X		
		unidentified	X	X	
	Thysanoptera	Phlaeothripidae	X	X	
		unidentified Terebrantia		X	
	Psocodea	unidentified	X	X	
	Hymenoptera	Agaonidae		X	
		Apidae		X	

(*Continued*.)

Table 1. (*Continued*.)

class	order	family or group	nest	ground	notes
		Braconidae	X		
		Eulophidae	X		
		Formicidae: Dolichoderinae	X	X	
		Formicidae: Formicinae	X	X	*Camponotus, Polyrhachis*
		Formicidae: Myrmicinae	X	X	*Crematogaster, Cataulacus, Monomorium, Strumigenys*
		Formicidae: unidentified	X	X	
		Platygastridae s.l.	X	X	includes Scelionidae
		Pteromalidae		X	
	Coleoptera	Carabidae	X	X	
		Chrysomelidae	X	X	Alticini
		Curculionidae	X		including Scolytinae
		Lycidae		X	larvae only
		Silvanidae	X		*Airaphilus*; found in four different nests
		Staphylinidae		X	
		Tenebrionoidea		X	
		unidentified		X	
	Diptera	Cecidomyiidae	X	X	
		Ceratopogonidae[a]	X	X	larva from ground; adults from nest; larva and one adult in Forcipomyiinae
		Chironomidae		X	
		Chloropidae	X		
		Drosophilidae	X		
		Hybotidae		X	
		Phoridae		X	
		Psychodidae[b]	X		Phlebotominae
		Sciaridae	X	X	
		Tipulidae		X	
		unidentified		X	
	Lepidoptera	unidentified	X	X	single unidentified moth from nest

[a]Denotes taxa that potentially feed on blood (ectoparasites).
[b]Denotes taxa that feed on blood (ectoparasites).

beds relative to branches or leaves of the same tree ($\chi^2 = 1.649$, $p = 0.199$). Similar to the case for faeces, skin-associated bacteria were no more common in chimpanzee beds ($\chi^2 = 0.154$, $p = 0.695$; 2.4% of total reads) than in environmental samples. Particularly noteworthy was that, although *Corynebacterium* is the most abundant skin-associated taxonomic group currently described from chimpanzees (as well as from gorillas) [61], we found no *Corynebacterium* in chimpanzee beds. Oral bacteria, on the other hand, were more abundant in chimpanzee beds than on adjacent branches and leaves ($\chi^2 = 14.644$, $p < 0.001$). However, these too represented a very small portion of the total abundance of all microbes (0.82% of sequence reads from beds, 0.03% of sequence reads from the environment). Collectively, body-associated taxa (be they faecal, skin or oral in origin) accounted for only 3.5% of all microbial sequence reads from within chimpanzee beds.

3.2. Arthropods

Arthropods were more abundant on the ground than in chimpanzee beds ($p = 0.007$; $n = 226$ ground specimens, $n = 108$ bed specimens; table 1). Nonetheless, beds ($n = 15$) were host to 12 orders of

arthropods, comprised 47 total morphospecies, with an average of 5.2 orders and 3.1 morphospecies represented per individual bed. Of all morphospecies collected just two are known ectoparasites of mammals (Phlebotominae and Ceratopogonidae, $n = 3$). All three specimens from these families were collected from within beds. We also collected one specimen of a potential blood-feeder from the Anthocoridae family ($n = 1$; table 1). We collected one Ceratopogonidae larva from the ground below a chimpanzee bed; however, though the adults of Ceratopogonidae are blood-feeders, the larvae are not, so this specimen was not included in the total number of ectoparasites.

Of all arthropods collected within beds, none was from a lineage known to be strongly dependent on chimpanzees or mammal structures [65,66]. One potential exception was that of the silvanid beetles (Silvanidae). These beetles are often found in human homes [66]; however, after further identification, we found that the silvanid beetles collected from chimpanzee beds belonged to the genus *Airaphilus*. The beetles within this genus feed on fungal spores and dead plant material and are commonly found beneath the bark of dead trees or in leaf litter. Owing to their ecological niche, it is unlikely to be a group directly associated with chimpanzee bodies or structures ([67], Dr M. C. Thomas 2016, personal communication).

4. Discussion

The exposure of a mammal to pathogens, environmental bacteria, insects and other sympatric taxa is likely to be strongly influenced by the ecology of its sleeping place. We hypothesize that this has been the case for tens of millions of years, such that mammalian immune systems have evolved in the context of frequent exposure to environmental species. It has often been suggested that we have reduced the diversity of our exposures, as we have begun to spend more time indoors. Yet, though it has become increasingly clear that which species mammals, including humans, are exposed to can have both beneficial and detrimental effects on health and well-being, little is known about what those interactions might have been historically, or how such interactions vary among our living relatives. Here we present, to our knowledge, the first study of the organisms found in the sleeping place of a non-human mammal, that of wild chimpanzees.

Based on the study of human homes [36], one might hypothesize that the microbes found in chimpanzee beds would be less diverse than that of the adjacent environment, and further, that chimpanzee beds would be primarily composed of body associates. Instead, we found that the diversity of bacteria in chimpanzee beds was similar to that of the surrounding environment (electronic supplementary material, table S3*a*). In addition, taxa from chimpanzee bodies were almost entirely lacking in beds. Though we recognize that there is still more research needed on the characterization of microbiomes from wild chimpanzees, the near complete absence of currently defined body-associated taxonomic groups from within chimpanzee beds indicates that there is likely to be little accumulation of such species. The construction and likely inhabitation of a bed influenced which bacteria were present; however, the season in which each bed was built and the elevation above sea level explained most of the variation in microbial diversity and community assemblage (electronic supplementary material, table S3). Similarly, we found only four arthropod individuals known to be ectoparasites within beds, none of which appears to be a specialist on chimpanzees or their structures (table 1). In short, our results suggest that the microbes and arthropods to which chimpanzees are exposed while resting are predominately environmental, contingent upon season and location on the landscape.

The beds made by great apes, be they chimpanzees, gorillas, bonobos or orangutans, are typically used for a single night and then abandoned [22]. This movement of beds from one night to the next has long been thought to serve a range of beneficial functions. One explanation for such movement is that it decreases the ability of pathogens and pests to build up at a sleeping site and reduces the microbial odours associated with the individual that might attract predators [68,69]. Our results are commensurate with this hypothesis, as we found little evidence of the accumulation of bacteria or arthropods in chimpanzee beds. The lack of faecal bacteria may also be owing to chimpanzee toilette hygiene. Chimpanzees usually defecate over the sides of their beds [70]. Our data suggest they are effective at doing so in a way that prevents soiling the beds themselves. In addition, we found no arthropods in beds that were closely associated with chimpanzees and only four mobile blood-feeder specimens. Yet, chimpanzees are host to more than 60 parasites and pathogens, including lice and fur mites [65,71,72]. Given this, our results may reflect effective grooming practices (such as consuming ectoparasites), which prevent those species from reaching high abundances even when present. These findings highlight the need for more research on wild, habituated primate populations which would allow for the direct collection of microbes and arthropods from individuals and access to beds immediately following

abandonment. We could then more fully explore the strength of individual variation, as well as directly observe behaviour within beds, which was not possible within the scope of our study.

4.1. Invention of the indoors

Though chimpanzees are not human ancestors, having diverged from a common ancestor between 6.6 and 12 million years ago [73,74], the building of beds by great apes is an ancestral trait that is thought to have appeared before the divergence of the hominid and hominin lineages [21–22,24]. Chimpanzees have often served as a model for reconstructing the behaviour of early hominin species [75–79], including the evolution of structure building [24]. Furthermore, it has been hypothesized that early hominins built beds in which to rest, as is seen among modern great apes [79–82]. Based on the reconstructed history of building among these groups, the beds of chimpanzees are likely to share common features with those of our hominin ancestors, especially given that our ancestors exhibited morphological adaptations for arboreality (*Ardipithecus ramidus*, [83]; *Australopithecus afarensis*, [84]; *Homo habilis*, [85]) and may have moved from sleeping site to sleeping site, as has been argued [37,81]. In as much, chimpanzee beds offer a window into the potential exposures of our ancestors while sleeping, even if an imperfect one.

Chimpanzee beds and human homes share two of the three most abundant microbial phyla (Proteobacteria and Actinobacteria). However, this similarity hides major differences in the likely origins of these microbes, differences that can be better seen if we consider the taxonomic level of families. Methylocystaceae, Pseudonocardiaceae and Microbacteriaceae were common in chimpanzee beds and are all previously described environmental microbes and/or soil associates [86–88]. By contrast, the most abundant families of bacteria in human homes are those associated with human skin or faeces; Streptococcaceae, Corynebacteriaceae and Lactobacillaceae [36]. To put it simply, we have created sleeping places in which our exposure to soil and other environmental microbes has all but disappeared, and we are instead surrounded by less diverse microbes that are primarily sourced from our own bodies [36,89]. The situation is similar with regard to arthropods. Chimpanzee beds contained no arthropod specimens specialized on life with chimpanzees. By contrast, the arthropod communities in human homes are diverse, often including hundreds of species, tens of which are specialized on life indoors with humans [6,66].

We do not yet know enough to reconstruct the complete history of human sleeping places and the species that composed their communities. However, we can propose based on our results from chimpanzee beds that at some point in hominin evolution, probably no earlier than a million years ago [90–91] and no later than 20 000 years ago [1–2], our ancestors made a major transition in terms of their exposures to other organisms while sleeping. They began to sleep repeatedly in the same spots and, in doing so, provided the opportunity for recurrent exposures to the subset of species that live on bodies and in beds and homes. With that change, the proportion of time we spend with these species has continued to increase, as we now spend the majority of our lives indoors. Meanwhile, our exposure to environmental microbes and arthropods has decreased. If true, exposure to our own microbes and to the arthropods adapted to the human built environment may be novel, relative not only to our recent history but also potentially to our more ancient past.

Ethics. No data were collected from human or animal subjects for the purpose of this study. All chimpanzee beds were sampled following site abandonment, and there was no direct contact with any of the chimpanzee individuals.

Data accessibility. Microbial data from this project were deposited in the Dryad data repository and made publically available at http://dx.doi.org/10.5061/dryad.7cp50 [92].

Authors' contributions. M.S.T. designed collection protocols, coordinated sample processing and conducted statistical analyses; F.A.S., R.A.H.-A., N.C. and A.K.P. coordinated and conducted field data collection; A.K.P. obtained collection permitting; R.R.D. conceived of and F.A.S., R.A.H.-A. and R.R.D. designed this research project, M.A.B. identified all arthropod specimens; D.A.B. designed protocols and conducted extractions for microbial samples; R.J.B. contributed resources and laboratory space; K.P.C. processed microbial sequencing data and aided in the interpretation of results. The manuscript was drafted by M.S.T., F.A.S., R.A.H.-A., A.K.P., M.A.B., D.A.B., K.P.C. and R.R.D. All authors gave final approval for publication.

Competing interests. We have no competing interests to declare.

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CHAPTER 3: The Himba Home: Insights into Global Household Ecology from the Homes of Pastoralists

Prepared for publication

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Abstract

Approximately 10,000 years ago, humans began to transition from a primarily hunter-gather lifestyle to one that included raising livestock and tending agricultural crops. During this time, we moved from open, ephemeral structures to seasonal homes constructed from mud and manure, and since the rise of pastoralism, we have increasingly settled into more permanent homes and sealed ourselves from the outdoors. Though there is mounting evidence that changes in our architecture over time have greatly influenced our species interactions, and in turn our health, our associate communities from this period in human history have yet to be fully characterized. Here, we worked with the Himba community and residents of the United States to compare differences in bacterial communities between modern pastoralist homes in Namibia (n = 5) and contemporary homes in the United States (n = 13), where we focused on three habitats from each home (an exterior door sill, an interior door sill, and a pillow). We then combined our results with data from previous studies to consider how bacterial communities from pastoralist and contemporary homes compare within a broader range of built environments. These include studies from chimpanzee nests (nightly structures similar to those built by early humans) and the International Space Station (ISS), an environment that is completely closed with no exposure to the outdoors. We found that although the bacterial diversity on the exterior of Namibian and United States homes was approximately the same, homes in Namibia had a much higher diversity of bacteria on indoor surfaces. In addition, whereas we saw no difference in the overall assemblage of bacteria among Namibian home habitats, homes in the United States showed strong differentiation and distinct bacterial communities dependent upon surface sampled. When we considered the source of those bacteria, Namibian homes had slightly more soil-associated bacteria (a group that is important for immune development in humans), where homes in the United States had approximately twice the relative abundance of bacteria associated with the

human body, especially from the skin and mouth. Namibian homes had a greater abundance of fecal bacteria; however, we could not differentiate between feces from humans and other mammals. Unlike the homes we sampled in the United States, Namibian homes do not have bathrooms indoors, so it seems likely that fecal associates we detected are from home construction materials (i.e., manure) and/or the Himba's close association with cattle and other livestock. When we then compared our data to those from other studies, we found similar patterns. As structures progressed from the least modified (chimpanzee nests) to complete removal from the outdoors (the International Space Station), we found progressively less soil bacteria and a greater abundance of bacteria from the body, where skin associates showed the strongest response compared to bacteria from the mouth and feces. Our results suggest that through some combination of lifestyle and home design, our species interactions within the built environment have shifted predictably over time. However, although we do see significant differences in the diversity and composition of bacteria found in Namibian and United States homes, perhaps the biggest transition in our species interactions happened when we moved from open, ephemeral structures (similar to those built by chimpanzees) to more permanent homes (similar to modern pastoralist houses). In addition, as we continue to further remove ourselves from the outdoor environment, it is likely that our exposures to diverse environmental microbes, including those that are important for immune development (i.e., soil associates), will decrease to a greater extent, and we will instead be surrounded by more bacteria sourced from our own bodies.

Introduction

Since the proposal of the hygiene hypothesis (Strachan, 1989), the relationship between human health and our exposure to diverse environmental species has become increasingly

apparent (Hanski et al., 2012; Ege et al., 2011; Rook, 2013). We now know that our microbiome is directly altered by the bacteria in and around our homes, and a shift in the types and diversity of those species over time has, in some regions, negatively impacted our physical health and mental well-being (Hanski et al., 2012; Haahtela et al., 2015; Rook et al., 2008; Hoisington et al., 2015). For example, recent studies have shown that common soil bacteria are more abundant on the skin of individuals that live closer to forests and agricultural fields. When people then lose these exposures to soil associates, such as *Acinetobacter*, they are more likely to develop autoimmune disorders and allergies (Ruokolainen et al., 2015; Fyhrquist et al., 2014). Additionally, contemporary houses favor some pathogenic lineages, such as *Legionella* spp. and non-tuberculous mycobacteria (associated with hot water and showerheads; Wadowsky et al., 1982; Feazel et al., 2009), and promote the accumulation of human, body-associated microbes (Dunn et al., 2013; Lax et al., 2014). Yet for much of our evolutionary history, it is likely that our species interactions were defined not by the microbial taxa favored within our built environment, but rather by the species we came into contact with as we hunted for and collected our food, tended to agricultural crops, and raised livestock. Changes in our exposures, relative to the exposures of our ancestors, are likely to accelerate as we become more concentrated in urban centers and as our buildings are increasingly sealed off from the outdoors (Martin et al., 2015). However, it is not well understood how the species associated with contemporary homes compare to those in more traditional homes or how those two home environments fit within the broader context of the evolution of the indoor biome.

While traditional homes around the world vary greatly (Panagiotakopulu, 2003; Jarzombek, 2014) compared to contemporary homes, they also share some common features. Traditional homes tend to be small (relatively few square feet per person), less compartmentalized (fewer discrete rooms), and built of harvested rather than engineered

materials (e.g., wood, mud, and leaves). In addition, they often have windows and doors that do not completely disconnect the inside and outside world. Indoor climatic variations are therefore due to passive heating and cooling, rather than mediated by mechanical ventilation systems. This feature alone has the potential to strongly influence which species are found on surfaces, as open ventilation decreases the accumulation of pathogens and increases the frequency of contact with diverse microbial communities, promoting healthy immune development, particularly in children (Klemola et al., 2004; Ege et al., 2011; see Fink, 1985 and Reed, 2006 for historic examples). For communities that rely heavily on domesticated animals, whether animals allowed to move around and even into homes or those whose parts are used in home construction, the microbes from those animals might also play an important role indoors.

Currently, much of our understanding of the diversity of species in traditional homes comes not from ecology, but instead from archaeology. Studies of metazoan parasites in archaeological sites indicate a greater abundance of intestinal worms (helminths) associated with an increase in the size of villages and the permanence of houses (Hugot et al., 1999). These helminths then become rarer in contemporary homes in the last two hundred years, due to changes in water supply treatment, the use of shoes, and other public health interventions (Bleakley, 2007). In other words, exposure to worms shows a hump-shaped pattern with regard to settlement size and Westernization. Insects found in archaeological sites, on the other hand, often include many of the very same species found in contemporary homes (Panagiotakopulu, 2003), albeit with greater abundance and a few exceptions. For example, the parasites of domestic animals that were once very common in homes disappear with removal of their hosts. Also, grain pests are more common in homes located within agricultural settlements (Dugmore et al., 2005; Panagiotakopulu & Buckland, 1991) and less common in homes in societies in which grain storage occurs in silos far from homes. In addition, carrion and dung beetles were common

in historic homes (and may still be in some regions) due to the lack of sanitary waste disposal (Panagiotakopulu, 2001). For bacteria and other microbes, however, the potential contamination of archaeological sites is too great to make samples from such sites useful, and therefore, we must use referential models to infer historical species interactions. Fortunately, a small set of studies has begun to consider the microbiology of traditional homes.

In the most extensive study to date on traditional homes, Ruiz-Calderon and colleagues (2016) investigated homes in the Brazilian Amazon ranging from open, traditional homes to apartments. They found that traditional homes tended to differ in the composition of their bacteria from contemporary homes, with a greater abundance of environmental, as opposed to human, microbes. The simplest expectation for other traditional homes, be they of hunter-gatherers, pastoralists or even farmers, is that their indoor communities will also be predominantly composed of environmental microbes rather than human associates and more similar to each other than to contemporary houses.

Our focus here is on traditional pastoralist homes built of natural materials, but in which domesticated animals are a prominent feature of daily life. We focus on one community of Himba pastoralists in Namibia. The ancestors of the Himba were Bantu agriculturalists who spread into the region that is now Namibia, roughly five hundred years (Vogelsang et al., 2002; Barbieri et al., 2014). Upon colonizing the region, Himba ancestors mostly abandoned crop-based agriculture and now live a lifestyle dominated by tending cattle, goats, and sheep. The Himba live in small familial villages and move from place to place with the wet and dry seasons. A typical Himba village is likely to have two styles of houses, both of which are framed by branches and covered with mud and manure. In one style, the branches are bowed to form a single dome (Figure 1). In the other, a set of branches forms a vertical (and circular) wall. On top

of those branches another set of branches forms a roof that comes together at a central point (Jarzombek, 2014).

We worked with members of the Himba community in Namibia and residents of the United States (US) to sample bacteria from three habitats in their homes (similar to the approach used in a previous US study; Dunn et al., 2013). Based on the diversity hypothesis (Hanski et al., 2012) and empirical results from the study of Brazilian homes (Ruiz-Calderon et al., 2016), we predict traditional pastoralist houses in Namibia to have a greater diversity of bacteria compared to contemporary Western homes. In addition, due to their small size, lack of compartmentalization, and greater continuity between the indoors and outdoors of Namibian homes, we hypothesize that pastoralist houses will have less differentiation of bacterial communities among surface habitats, a greater relative abundance of soil- and livestock-associated bacteria, and less accumulation of body associates compared to homes in the United States (Lax et al., 2014; Adams et al., 2014; Miletto & Lindow, 2015; Adams et al., 2016). Finally, we compare our results from Namibian and United States homes to previous studies that represent a range of built environments, including chimpanzee nests (similar to the open, ephemeral structures built by ancient humans; Thoemmes et al., 2018) and the International Space Station (an indoor environment that is completely sealed from the outdoors; Lang et al., 2017).

Materials and Methods

We had participants of the Wild Homes citizen science research project (http://robdunnlab.com/projects/wild-life-of-our-homes/) sample three standardized locations in homes in Namibia (n = 5) and the United States (US; n = 13), using sterile BBL™ CultureSwabs™: an upper exterior door sill, an upper interior door sill, and a pillow. We

selected these sample locations as they were readily identifiable for all home types, and Dunn et al. (2013) found their microbial communities to be statistically different in a previous study of US homes. This research was approved by the North Carolina State University's Human Research Committee (Approval No. 2177).

Microbial Analyses

We cut swab tips into a 96-well, deep well plate and extracted DNA with the MoBio PowerSoil HTP Soil DNA Isolation Kit, with modifications as described in Fierer et al. (2008). We amplified the V4/V5 region of the 16S rRNA gene from each sample in triplicate, using the 1x5 PRIME Hot Master Mix kit (5 PRIME Inc.) and reaction conditions described in Flores, Henley & Fierer (2012). We then quantified amplicons from each triplicate reaction using a PicoGreen dsDNA assay (Life Technologies) and pooled replicates in equimolar concentrations before sequencing on the Illumina MiSeq platform at the University of Colorado Boulder, Next-Generation Sequencing Facility, CO, USA. Details on sequencing approach are available in Caporaso et al. (2012).

We joined paired-end reads, demultiplexed our data, and quality filtered sequences with default parameters in the QIIME, v1.9.1 pipeline (Caporaso et al., 2010). We picked OTUs using UCLUST at a 97% similarity and assigned taxonomy with the RDP Classifier (Edgar, 2010; DeSantis et al., 2006). We then removed all sequences classified as mitochondria, chloroplasts, and unassigned taxa and quality filtered our data using the Bokulich threshold, in which we removed all OTUs that represented less than 0.005% of total read abundance (Bokulich et al., 2013). This method accounts for error replication during PCR reactions (Nguyen et al., 2015), and it has been shown to be the optimal method for the removal of contaminant OTUs within environmental samples (Krohn et al., 2016). We rarefied the remaining data to 6000 sequence

reads per sample. One sample from an exterior door sill for a US home did not meet the rarefication threshold. We analyzed all remaining data with the mctoolsr and vegan packages within the R environment (Leff, 2016; Oksanen et al., 2013; R Core Team, 2015).

We tested differences in OTU richness between Namibian and US homes, as well as among surface habitats with Kruskal-Wallis tests. We considered the assemblage of bacteria and visualized community composition data with non-metric multidimensional scaling (NMDS) ordination plots and tested the overall divergence of those communities between Namibian and US homes with an analysis of variance (ANOVA). Furthermore, as there was a strong differentiation among habitats in a previous US homes study (Dunn et al., 2013), we considered the differentiation of bacterial communities among Namibian home surface habitats with a permutational multivariate analysis of variance (PERMANOVA), based on 999 permutations (Anderson, 2001). We then calculated the most abundant taxonomic family groups and tested the difference in the relative abundance of Micrococcaceae between Namibian and US homes, as this group is one of the most abundant skin associates of mammals, including common livestock species (Dastager et al., 2014). For Micrococcaceae, we included exterior surfaces in our analysis, since geographic region is a determinate of which species colonize the outside of buildings (Grantham et al., 2015; Barberán et al., 2015) .

Source-tracking

We quantified the percent relative abundance of bacterial taxonomic groups thought to have been lost or acquired on indoor surfaces as we became more isolated from the outdoor environment (i.e., soil and body associates) and tested all differences in associate groups with Kruskal-Wallis tests. For our analyses, we included soil taxa that were found to be both highly abundant and ubiquitous among globally diverse soils (n = 541 unique OTUs; Delgado-

Baquerizo et al., 2018), as well as *Acinetobacter* spp. For body associates, we quantified the relative abundance of human-associated taxonomic groups and compared overall differences between Namibian and US homes, as well as differences in the contribution of skin, oral, and fecal bacteria separately. In Namibian homes, we also compared differences in the abundance of body associates among surface habitats (i.e., the exterior door sill, interior door sill, and pillow). We used skin, oral, and fecal bacteria defined in Dunn et al. (2013), as well as fecal bacteria characterized by Arumugam et al. (2011; i.e., *Parabacteroides*, *Faecalibacterium*, *Bifidobacterium*, *Collinsella*, and *Eggerthella*). Also, as fecal communities show strong and predictable differentiation based on diet and environment (Wu et al., 2011; Obregon-Tito et al., 2015; Yatsunenko et al., 2012), we might expect variation in gut bacteria between Namibian and US populations. However, pastoralist gut bacterial communities have yet to be characterized; therefore, we chose to include gut associates that were previously described from the Hadza tribe (Schnorr et al., 2014). Though the composition of Hadza fecal bacteria may not exactly reflect those of the Himba, we would expect them to be a closer representation than that of people from industrialized nations. Both groups live in comparable savannah habitats (O'Connell et al., 1988; Bollig, 1997), and rural communities show similar patterns of gut microbial biodiversity (De Filippo et al., 2010; Obregon-Tito et al., 2015). Taxonomic families described among the Hadza include Ruminococcaceae, Lachnospiraceae, Prevotellaceae, Clostridiales Incertae Sedis XIV, Succinivibrionaceae, Spirochetaceae, and Eubacteriaceae. Ruminococcaceae and Lachnospiraceae were shared with our other fecal dataset. In addition, as the Himba women use ochre pigments to coat their skin and hair, we calculated the abundance of iron-oxidizing bacteria involved in ochre formation. We chose only the genera found to be universally present among previously sampled locations; *Gallionella*, *Sphaerotilus*, and *Leptothrix* (Wheatley, 1988).

The Built Environment

To understand how the evolution of built environment has altered our species interactions, we combined our data with previously published bacterial datasets, including from chimpanzee nests (Thoemmes et al., 2018), additional data from contemporary homes in the US (Dunn et al., 2013), and from the International Space Station (ISS; Lang et al., 2017; Supplementary Table S1). For each study, we downloaded data files from their respective repository, demultiplexed sequence reads, and quality filtered each file according to default parameters within QIIME. We merged all FASTA files and picked OTUs using a closed reference approach to accommodate different variable regions of the 16S rRNA gene (Rideout et al., 2014; Delsuc et al., 2014; McKenney et al., 2018). We then removed all OTUs designated as mitochondria, chloroplasts, and unassigned taxa. Due to a lack of analog from other projects, we removed kitchen surface samples from the previously published Wild Homes dataset (i.e., the refrigerator, cutting board, and kitchen counter samples; Dunn et al., 2013). Also, as we only have interior data from the ISS, and our primary goal was to assess how microbial communities found inside homes have changed (or are likely to change as our buildings become more modified), we included only data from interior surfaces or, in the case of chimpanzee nests, the locations that were likely to have direct contact with an individual's body. We rarefied these data to the same depth as our unique dataset, 6000 reads per sample, and visualized and compared differences in community composition among all datasets with the same methods as outlined above. To determine the predominate sources of bacteria in each home type, we calculated the percent relative abundance of skin, oral, and fecal bacteria, in which we used chimpanzee-associated taxa defined in Thoemmes et al. (2018).

Results

When we compared bacterial diversity between home types, OTU richness was greatest in Namibian homes ($\chi^2 = 27.439$, $P < 0.001$; Figure 2a). Each Namibian home had, on average, more than twice the number of bacterial OTUs (1690, n = 15) as did homes in the United States (723, n = 33). These differences were not due to regional variation in the outdoor species pool. Indeed, exterior door sills in Namibia were similar in bacterial diversity to those in the United States (average OTU richness = NM: 6405, US: 6269). The differences were instead due to the number of species that made their way indoors. Whereas the bacteria we collected inside Namibian homes were just as diverse as those on the exterior door sill (average OTU richness exterior: 1056 OTUs, interior: 1015 OTUs; $\chi^2 = 1.148$, $P = 0.284$), bacterial diversity declined inside US homes, with an average of 896 OTUs on the exterior door sills and 676 OTUs on interior surfaces ($\chi^2 = 4.284$, $P = 0.04$; Figure 2b). Diversity was lowest where there was the most contact with human bodies (i.e., pillows; $\chi^2 = 9.947$, $P = 0.002$).

In addition to differing in their diversity, homes also differed among countries in terms of the composition of their bacterial communities ($P < 0.001$; Figure S1). In Namibian homes, we found no differentiation among habitats ($P = 0.183$; Figure S1). The bacterial communities on pillows were similar to those on the inner door sill ($P = 0.203$), which were only modestly different from those of the outer door sill ($P = 0.1$). In the US, on the other hand, we saw a clear differentiation in bacterial community membership among habitats ($P < 0.001$; Figure S1).

When we compared the composition of taxonomic families among homes and countries, Micrococcaceae was the most abundant group recovered from Namibian homes (8.15% of sequence reads). In contrast, in the US, Staphylococcaceae, Corynebacteriaceae, and Streptococcaceae were the most common families (accounting for 22% of sequence reads), while

Micrococcaceae represented only 3.5% of sequence reads (significantly less compared to homes in Namibia; $\chi^2 = 6.488$, $P = 0.01$).

Source-tracking

Soil-associated microbes accounted for 33.3% of all sequence reads inside Namibian homes and 27.2% of sequence reads from inside homes in the US ($\chi^2 = 0.404$, $P = 0.525$; Figure 3). However, we observed a significantly higher abundance of soil bacteria of the genus *Acinetobacter* (associated with benefits to immune health) inside Namibian homes ($\chi^2 = 6.115$, $P = 0.01$), accounting for 3.8% of bacterial sequence reads, compared to only 1.6% of sequences among US homes. Body-associated microbes were approximately twice as relatively abundant in US homes (29.1% of sequence reads) as they were in homes in Namibia (14.2% of sequence reads; $\chi^2 = 4.795$, $P = 0.029$); however, those differences were dependent upon source (i.e., whether the bacteria were from the skin, mouth, or feces; Figure 3). Skin and oral microbes were less common inside Namibian homes than in homes in the US (average percent of sequence reads from skin: Namibia - 6.4%, US - 20%, $\chi^2 = 8.388$, $P = 0.003$; oral: Namibia - 2.1%, US - 9%, $\chi^2 = 10.79$, $P = 0.001$). Fecal microbes, however, were more abundant inside Namibian homes (Namibia - 7.3%, US - 4.1%; $\chi^2 = 9.014$, $P = 0.003$), though we could not distinguish bacteria associated with human feces from those associated with domesticated animals. Fecal bacteria represented an average relative abundance of 7% of sequence reads on exterior door sills and 7.29% of sequence reads on interior surfaces ($\chi^2 = 3.381$, $P = 0.066$), suggesting that at least some of these bacteria may be associated with the animals outside or the dung used in house construction. Based on the use of ochre on pillows (and skin) we predicted that iron-oxidizing bacteria might be more common on pillows than in other habitats. Yet, we found none of these taxa within Namibian homes, regardless of surface.

The Built Environment

The composition of bacterial communities found in chimpanzee nests, homes in Namibia, homes in the United States, and those on the ISS all differed significantly from one another (R^2 = 0.35, $P < 0.001$; Figure 4). Chimpanzee nests showed the greatest amount of variance in microbial membership from one sample (nest in this case) to the next, followed by US homes and the ISS (Figure 4). Namibian homes showed the least variation in microbial membership. Differences in built environment type become even more apparent when we consider the source of bacteria. As structures become more modified, permanent, and closed off from the surrounding environment, there is a decrease in the relative abundance of soil bacteria and a subsequent increase in body-associated groups (Figure 3). Overall, our results suggest that types of dwellings and nests can be arranged in a continuum. At one end of the continuum are chimpanzee nests, in which virtually all bacteria present are associated with soil or the environment. At the other end of the continuum are a subset of Western, architect-designed homes dominated by body-associated bacteria. In between these extremes we find the Himba homes, homes that until very recently resembled the vast majority of homes on Earth, in as much as they are small, relatively open to the outdoors, and occupied only part of the day (Figure 3).

Discussion

Overall, there were differences in the diversity and composition of bacteria between pastoralist homes in Namibia and contemporary homes in the United States. The average Namibian home had diverse communities that showed no differentiation among sampled surfaces. In contrast, US homes had less bacterial diversity indoors, relative to the outdoor environment, and distinct bacterial communities dependent upon surface habitat. In addition, we recovered a greater abundance of environmental bacteria (i.e., bacteria from the soil) and fewer

body-associated taxa from Namibian homes than from homes in the US. When we considered traditional pastoralist homes and contemporary Western homes within the broader context of the evolution of the built environment, we observed similar trends. The more highly modified and removed from the outdoors a home or nest was, the less abundant soil bacteria were on interior surfaces and the greater the accumulation of body associates (particularly those of the skin; Figure 3).

It has become increasingly apparent that our exposure to a diverse community of microbes in our homes and buildings is important for individual health (e.g., Strachan, 1989; Hanski et al., 2012). However, the magnitude of this loss over time is less well-understood. Here, we are not comparing the past to the present. We are, however, able to compare homes like those in which many people on Earth still live (small, relatively open homes with passive heating and cooling) to Western homes that are designed to be climate controlled and sealed off from the outdoors. Such homes share many features with historic homes, and so, offer hints as to the sort of change that might have occurred through time. Here, find that the pastoralist homes of Namibia have approximately twice the diversity of bacteria indoors compared to contemporary US homes. Furthermore, whereas there was no difference in the OTU richness on the exterior and interior of houses in Namibia, there was a strong decline in bacterial diversity as you move further indoors (from door sills to pillows) in US homes. In addition, Namibian homes had little compartmentalization among surface habitats. The Himba homes in Namibia act more like a singular microbial environment, or in other words, a single habitat. This could be due to the open design of pastoral homes, which results in greater microbial exchange with the outdoor environment; however, their smaller size and lack of division into individual rooms are also potential factors.

A large body of literature now argues that as humans, in some regions (such as Northern

Europe and the United States), have moved into more modified environments, they have lost key associations with other species (Haahtela et al., 2015; Ruokolainen et al., 2015; Stein et al., 2016). Some of these associations are likely to be general and broad (e.g., plant-associated microbes), but it is also possible that our location on the landscape and geographic region have played an important role in how our species interactions have shifted over time. The Himba live in an environment similar to that in which our human ancestors lived for hundreds of thousands of years, both in terms of the basic features of the construction of houses and in terms of the environmental conditions in which the houses are built (Bobe, 2006; Feakins & Eglinton, 2005). For instance, the plants in the Himba environment may include many of the same plant lineages consumed by early *Homo* species (Ungar & Sponheimer, 2011). In this light, we find it reasonable to ask if there are lineages of bacteria found in Himba homes that are absent from homes across North America, lineages that might have been lost from daily life with the transition from thatch and mud homes to high rises. And indeed, we did find different origins of bacteria. Micrococcaceae, a commensalistic lineage common on mammalian skin (Dastager et al., 2014), was more than twice as common in Himba homes than in US homes, relative to other OTUs. This may simply reflect the more direct connection between the Himba home environment and the people that inhabit them - bodies that are less covered by shirts, pants or shoes. However, this interpretation is at odds with the relative lack of accumulation of other body microbes (e.g., Staphylococcaceae, Corynebacteriaceae, and Streptococcaceae). Alternatively, the high relative abundance of Micrococcaceae may be due to continuity between indoor and outdoor environments, an effect of frequent contact with cattle and other livestock, along with the use of dung in home construction.

In contrast with our predictions, we found only a slightly higher relative abundance of soil bacteria in Namibian homes compared to homes in the United States. Instead of a general

loss of soil bacteria, we found that a few key taxa appeared to be rare in homes in the United States. For example, *Acinetobacter*, which has been shown to be vital to the development of immune response in humans (Ruokolainen et al., 2017; Fyhrquist et al., 2014), was much more abundant in Namibian homes than in homes in the United States. In a previous study, the abundance of *Acinetobacter* on the skin was directly correlated with the proximity of a home to forests and agricultural fields (Hanski et al., 2012); therefore, the decrease in accumulation of this taxa in contemporary homes may be less explained by the general filtering of diverse microbes, but rather, it is possible that our homes are differentially colonized by specific bacterial groups as our cities have become more urbanized. This highlights the need for more research in these areas. We have not yet characterized the full range of species (or species interactions) that affect an individual's immune development and overall health or how our city and architectural design may influence our rates of exposure to those particular taxa.

In line with what we might expect given how open Himba homes are to the environment, body-associated microbes, including skin and oral microbes, were much less common in Himba homes than they are in homes in the United States (reviewed in Adams et al., 2016; Figure 3). We detected a high relative abundance of Staphylococcaceae and Streptococcaceae in US homes, which is consistent with the findings from previous studies (Adams et al., 2014; Miletto & Lindow, 2015; Barberán et al., 2015). This lesser accumulation of skin and oral associates in Himba homes could be an artifact of the ephemeral nature of their dwellings, which might not allow for the accumulation of bodily taxa over time. Or, more likely, differences in body associates between Namibian and US homes could result from a relative increase in the diversity of other taxa (i.e., through competition from environmental species). We believe this to be the more likely scenario, as we shed millions of bacteria from our bodies every hour (Qian et al., 2012), and microbial communities in homes shift in response to a new inhabitant within 48 hours

of occupation (Lax et al., 2014), suggesting there is a strong contribution of body groups on interior surfaces. Alternatively, fecal bacteria composed a greater proportion of bacteria in Himba homes. Close proximity of livestock to living areas, the use of manure in building construction, and little to no use of water for cleansing and sanitation could all be potential contributing factors. Further, the greater relative abundance of fecal bacteria could "flood" both exterior and interior habitats with fecal microbes, thus masking our ability to detect skin and oral microbial taxa.

Finally, we consider our findings in light of a broader range of built environments, from those that are completely exposed (i.e., chimpanzee nests) to those that are fully removed from the outdoors (i.e., the International Space Station). In general, we see evidence for a gradient in the composition of bacteria in different kinds of homes, beds and dwellings. At one extreme is the International Space Station which appears to be almost exclusively dominated by body bacteria. At the other extreme are the microbiomes of chimpanzee nests which are comprised nearly entirely of environmental bacteria. Our data from Namibian and US homes suggests that different home styles around the world exist at different points along this gradient. We hypothesize that the more we seal off homes, the more they become like the International Space Station in having a less diverse microbial community dominated by human associates. Himba homes, like many traditional homes around the world, are not fully open to the environment and so, include some body associates and many environmental microbes. It seems likely then this was also true for the first human homes, such that our exposures for tens of thousands, and maybe hundreds of thousands, of years might have been similar to those of the Himba. Then, over the last few hundred years, we began to build a new kind of home - one relatively tightly sealed off from the environment (and ever more like the International Space Station). Furthermore, in terms of the exposure of individuals to body microbes, the first homes were

likely to have been relatively novel in the history of mammals. The homes now being built in the United States and elsewhere have even less precedent in terms of the exposures they offer to their inhabitants, particularly when one realizes that we now spend more than twenty-three hours a day indoors (Klepeis et al., 2001). Such changes have health consequences, consequences we are just beginning to understand and that relate to the radical and unintentional shifts we have made to the species we sleep on, breathe in and come into contact with throughout our lives.

Ethics Statement

Each participant was provided with an Informed Consent form. Informed consent and research study were approved by the North Carolina State University's Human Research Committee (IRB Approval No. 2177).

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Tables and Figures

Figure 1. One of the two styles of Himba homes included in our study.

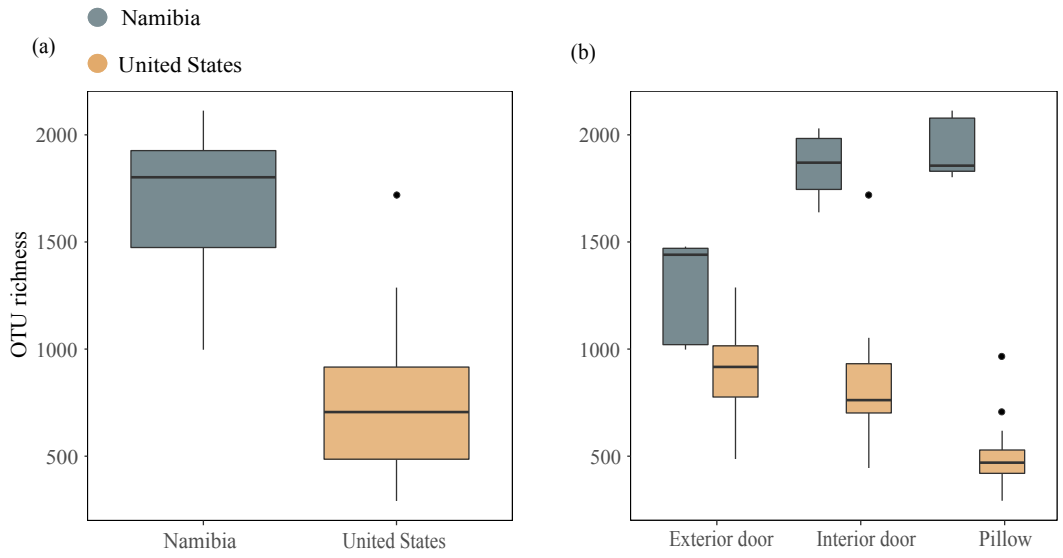

Figure 2. OTU richness from Namibian and United States homes *(a)* including all exterior and interior surfaces samples. *(b)* by surface habitat. Namibian homes had approximately the same diversity of bacteria on exterior and interior surfaces ($P = 0.284$). United States homes, on the other hand, showed the greatest amount of bacterial diversity on the outside of homes, with a sharp decline as you move further indoors (differences in OTU richness between outdoor and indoor habitats: $P = 0.04$; between interior door sills and pillows: $P = 0.002$).

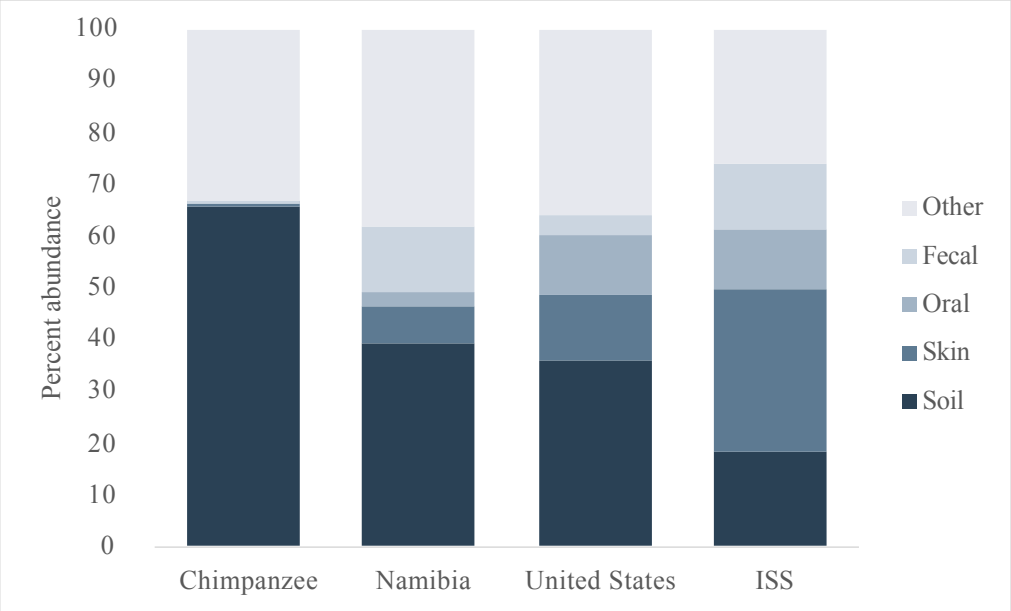

Figure 3. Average percent relative abundance of soil- and body-associated bacteria collected from chimpanzee nests, Namibian and United States homes, and the International Space Station (ISS). There was less accumulation of soil bacteria and a greater accumulation of body associates as indoor environments become increasingly sealed from the outdoors. Only indoor surfaces or, in the case of chimpanzee nests, the locations that were likely to have direct contact with an individual's body were included.

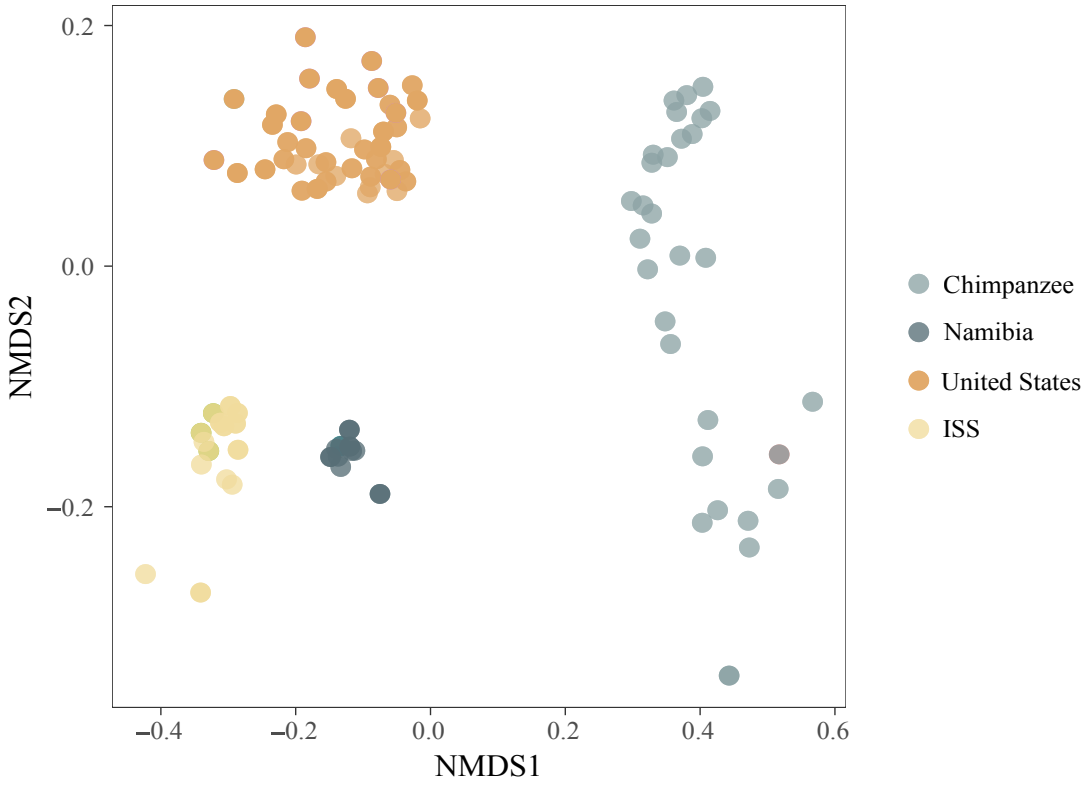

Figure 4. Non-metric multidimensional scaling (NMDS) ordination plot representing bacterial communities collected from chimpanzee nests, Namibian and United States homes, and the International Space Station (ISS). Overall, bacterial communities differed significantly, based on built environment type ($P < 0.001$), where chimpanzee nests showed the greatest amount of variation in community membership among samples. Differences among bacterial communities were calculated with Bray-Curtis dissimilarity metric, weighted by OTU abundance.

CHAPTER 4: Microbial Communities in the Natural and Supplemental Nests of the Endangered Key Largo Woodrat

Prepared for publication

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Abstract

Supplemental nests are often used in the conservation of threatened and endangered species, including the Key Largo woodrat (*Neotoma floridana smalli*). These structures are used to protect individuals from predation and provide a base on which additional nest building can occur. However, though their function mimics that of their natural counter-parts, their design is not fully representative of natural nests. Supplemental nests are enclosed and made from manufactured material (i.e., plastic culvert pipes), rather than downed wood and forest debris. Due to these differences in building design and material, the microbial species to which Key Largo woodrats are exposed might be altered, which could, in turn, negatively impact individual health. We predicted that supplemental nests would have less microbial diversity, a differentiation in overall microbial community membership, and a greater accumulation of particular taxa (e.g., pathogens), when compared to natural nests. To test this hypothesis, we sampled the bacteria from inside 10 natural and 10 supplemental Key Largo woodrat nests, as well as the forest floor (n = 10), in the Crocodile Lake National Wildlife Refuge. We observed distinct bacterial communities within Key Largo woodrat nests, relative to the forest environment; however, we could not differentiate between natural and supplemental structures based solely on the diversity and types of bacteria present. Supplemental nests also did not appear to favor rodent-associated pathogens. Natural and supplemental nests had approximately the same relative abundance of these bacteria, and where detected, we found them to occur at very low abundances. Where we expected to see an accumulation of pathogens, we instead found a high relative abundance of antimicrobial producing bacterial families, including Pseudonocardiaceae and Streptomycetaceae. In addition to nests, we sampled the bodies of the rodents that are known nest inhabitants, including native Key Largo woodrats, native Key Largo cotton mice, and exotic black rats (n = 30). The microbial biota of Key Largo woodrat

individuals resembled those of their nests, in that we observed an accumulation of Pseudonocardiaceae bacteria and a low relative abundance of pathogenic taxa. Other rodent species that use Key Largo woodrat nests, albeit less frequently, (i.e., the Key Largo cotton mouse and black rat) showed similar patterns. However, we found the overall composition of their body-associated microbial communities to be more divergent from Key Largo woodrat nests than those of the Key Largo woodrats themselves. Our results suggest that, although there appears to be some microbial interaction between nests and nest inhabitants, there are no detectable differences in the diversity and types of bacteria to which they are exposed within natural and supplemental nest structures.

Introduction

Supplemental nests are often used in the conservation of threatened and endangered species, as a way to increase available habitat and to provide protection from predators and the environment. Though supplemental nests model the function of their natural counterparts, they are commonly built from manufactured materials, with relatively little attention to mimicking the details of natural nest design. However, we know from the study of human homes that building material and architectural design strongly influence the diversity and types of microbes found on interior surfaces, and this has become ever more apparent as we have increasingly modified our indoor environments and attempted to seal ourselves from the outdoors. Contemporary houses have far fewer environmental microbes than do more open traditional homes (Thoemmes et al., In Prep), which, in turn, have fewer environmental microbes than do chimpanzee nests (Thoemmes et al., 2018). While the absence of some bacteria (such as *Vibrio cholera*) in our daily lives is beneficial, the absence of others is associated with negative health outcomes. For example, a decrease in the abundance of soil bacteria on the skin is directly linked to an increase

in the prevalence of atopic sensitization and autoimmune disorders (Ruokolainen et al., 2015; Fyhrquist et al., 2014). Additionally, as indoor microbial diversity decreases there is a subsequent increase in the abundance of bodily microbes, such as those from feces and skin (Dunn et al., 2013; Lax et al., 2014), as well as pathogenic bacteria in both homes and hospitals (Kembel et al., 2014). Despite this, no one has ever studied how human-built supplemental structures alter the microbial communities to which other mammals are exposed. A loss in microbial diversity or the accumulation of pathogens in supplemental nests could have detrimental effects, particularly for species at high risk of extinction, such as the Key Largo woodrat (*Neotoma floridana smalli*).

Key Largo woodrats are a federally endangered subspecies endemic to north Key Largo, FL (US Department of the Interior, 1984). Once estimated to number fewer than 100 individuals (McCleery et al., 2005), they have benefitted greatly from adaptive management, including nest supplementation (Cove et al., 2017). In fact, there have now been more than 2000 supplemental Key Largo woodrat nests built in the last remaining upland hammock habitat of the Crocodile Lake National Wildlife Refuge and Dagny Johnson Botanical State Park (Cove et al., 2017). Natural nests are typically built by layering sticks and debris from the forest floor at the bases of trees, in fallen tree throws, or in solution holes (a sink hole-like feature of the limestone bedrock of Key Largo created through erosion and acid rain; figure 1a). Supplemental nests, on the other hand, are constructed from large culvert pipes that are covered by rocks or chunks of fossilized coral (figure 1b). On the exterior, natural and supplemental nests are maintained in similar ways (i.e., through stick-stacking behavior; Cove et al., 2017), but it is on the interior of nests where differences become more apparent. Supplemental nests are enclosed with comparatively little air flow and moisture penetration (Barth, 2014). This is likely to limit the dispersal of environmental

species into nests and alter microclimate conditions, affecting the overall diversity and succession of microbial communities.

Here we consider the diversity and composition of bacteria in natural and supplemental Key Largo woodrat nests. Based on what we know from human homes, in which building design and material strongly influence the microbes that are found indoors, we might expect supplemental nests to have less bacterial diversity. In addition, as humans have sealed ourselves off from the outdoor environment, we have observed a shift in the relative abundance of microbial taxa (Adams et al., 2014; Miletto & Lindow, 2015; Barberán et al., 2015; Thoemmes et al., 2018), where we see an increase in the accumulation of body microbes and pathogenic bacteria (e.g., *Staphylococcus aureus*; Gandara et al., 2006). Since supplemental nests are composed from plastic materials that are likely to decrease airflow and restrict the colonization of environmental species in nests, we might then also expect there to be a difference in which bacterial taxa are most abundant based on whether a nest is natural or supplemental. Finally, as we know there a strong interaction between the species that live on bodies and in the built environment (Dunn et al., 2013; Hospodsky et al., 2012; Meadow et al., 2014; Gibbons et al., 2015; Lax et al., 2014), the composition of bacterial communities might be influenced by the nest inhabitants themselves. Therefore, we characterize the bacteria found on the bodies of the rodents known to use Key Largo woodrat nests, including Key Largo woodrats, Key Largo cotton mice, and black rats.

Methods

The Crocodile Lake National Wildlife Refuge is located in upper Key Largo, FL, USA. Though the majority of this refuge is composed of mangroves and coastal wetlands, it is the last remaining large tract of tropical hardwood hammock forest. When combined with the Dagny

Johnson Key Largo Hammock Botanical State Park, this forest type covers less than 1000 ha (Frank et al., 1997; US Fish and Wildlife Service, 1999) and is home to a variety of endemic and endangered species, including Key Largo woodrats, Key Largo cotton mice, and Stock Island tree snails (*Orthalicus reses*). Our study focused on the Key Largo woodrat, and we conducted all research in December 2017. This research was approved under the following permits: US Fish and Wildlife Service Permit TE 697819-4; Florida Department of Environmental Protection #01171715; North Carolina State University Institutional Animal Care and Use Committee (IACUC) #13-003-O.

Previously located natural nests and all supplemental nests are individually marked as part of a long-term monitoring project. From these, we visited 10 natural and 10 supplemental nests (n = 20) located in the area of the refuge that is most densely populated by Key Largo woodrats (Cove et al., *In review*). We determined nest occupancy based on visual surveys of active stick-stacking behavior (Balcom & Yahner, 1996; Cove et al., 2017) or additional evidence of use, such as with camera trap surveys or Sherman live-traps, and we swabbed each nest with dual-tipped sterile BBL™ CultureSwabs™. To standardize the distance into each nest, as well as to avoid contamination of sample swabs on exterior building material, we inserted a ¼" PVC pipe (approximately 0.5 m in length) into each nest prior to sample collection. We targeted areas that appeared to be used most frequently by the inhabitant(s) and then thread each swab through the pipe at the sample site. To determine how bacterial communities in nests vary, relative to the surrounding forest environment, we then swabbed the forest floor ~0.5-0.75 m from the exterior of natural nests (n = 10), in an area that did not appear to be trafficked by humans or wildlife. We swabbed all nest and forest floor environments for approximately 15 seconds.

In addition, as we know there is an interaction between mammal bodies and the structures they inhabit (Pakarinen et al., 2008; Hospodsky et al., 2012; Dunn et al., 2013; Hanski et al., 2012), we characterized the bacteria that live on the bodies of the rodents known to use Key Largo woodrat nests. We captured all individuals near sampled nests with Sherman live-traps and swabbed the flank and underside of 10 Key Largo woodrat, 10 Key Largo cotton mouse, and 10 black rat individuals (n = 30) for approximately 15 seconds. To prevent repeated sampling of individuals, we verified identity with double-marked monel ear and subcutaneous PIT tags.

Molecular Methods and Analyses

We performed DNA extractions with a DNeasy PowerSoil Kit (Qiagen, product #12888-100), with modifications described in Fierer et al. (2008). We included two sterile swabs at different stages in our extraction process to identify potential contaminants. PCR reactions were performed by the Fierer lab (University of Boulder, Colorado) in triplicate and all amplicons were pooled in equimolar concentrations prior to sequencing on the Illumina MiSeq platform, where we targeted the V4-V5 region of the 16S rRNA bacterial gene (Flores et al., 2012).

We processed all data using default parameters in the QIIME v1.9.1 pipeline (Caporaso et al., 2010) and picked operational taxonomic units (OTUs) with UCLUST at a 97% similarity (Edgar, 2010; DeSantis et al., 2006). Taxonomy was assigned with the RDP Classifier (Wang et al., 2007), and we removed all resulting OTU sequences from downstream analyses that were classified as mitochondria, chloroplast, and unassigned, as well as OTUs that were amplified from control samples (i.e., those from blank swabs and blank reagent wells). To quality filter our data, we used the Bokulich threshold, removing all OTUs that represented less than 0.005% of total read abundance (Bokulich et al., 2013; Nguyen et al., 2015). We then analyzed all data in

the R environment with the mctoolsr, vegan, PMCMRplus, and FSA packages (R Core Team, 2015; Leff, 2016; Oksanen et al., 2013; Pohlert & Pohlert, 2018; Ogle, 2018).

We compared differences in OTU richness and Shannon diversity index between nest and forest floor samples with Kruskal-Wallis, using the Dunnett's test and Benjamini-Hochberg method for multiple comparisons (n = 30; Dunnett, 1955; Benjamini & Hochberg, 1995). We then quantified differences in the composition of bacterial communities among samples with Bray-Curtis dissimilarity, weighted by OTU abundance (Bray & Curtis, 1957). We considered differences in bacterial community composition between natural and supplemental nests with a permutational multivariate analysis of variance (PERMANOVA), where we compared differences between nest type (i.e., natural and supplemental nests) and natural nest and forest samples separately.

We then tested for the presence of bacteria from genera known to contain zoonotic bacterial pathogens of wild rodents, as characterized previously by Razzauti et al. (2015). Though there is likely to be some variation in the pathogens found on Key Largo woodrats compared to other rodent species, these taxa encompass 45 bacterial genera, including those of well-known rodent-associated pathogenic groups (e.g., *Bartonella*, *Rickettsia*, *Borrelia*, *Neoehrlichia*, *Anaplasma*, and *Yersinia pestis*). Therefore, though we may have missed fine-scale interactions (e.g., previously undescribed pathogens or species-specific associations), we believe this representative dataset has captured general patterns in the accumulation of pathogenic bacteria in Key Largo woodrat nests. We then calculated the percent relative abundance of all pathogenic genera and compared differences between nest type and forest floor samples with Kruskal-Wallis tests.

To consider the bacteria from rodent bodies, we characterized the overall percent relative abundance of all bacterial taxa found in nests (n = 20) and on individuals (n = 30). In addition,

we compared the accumulation of bacterial taxa that contain pathogenic lineages in nests to those found on the bodies of Key Largo woodrats, Key Largo cotton mice, and black rats. Finally, we compared differences in community composition between nests and bodies with Bray-Curtis dissimilarity (Bray & Curtis, 1957) and visualized community data with non-metric multidimensional scaling (NMDS) ordination plots. We quantified differences with PERMANOVA, using an FDR correction for multiple comparisons.

Results

We observed a total of 1952 unique OTUs across all natural Key Largo woodrat nests, with an average of 776 OTUs represented per individual nest. This was no different from the OTU richness observed from supplemental nests (average of 919 OTUs per individual nest; $P = 0.41$) or from the forest floor (average of 841 OTUs per sample location; $P = 0.15$; Figure S1). We found similar results when we compared the Shannon diversity index, with no difference between natural and supplemental nests ($P = 0.56$) or between natural nests and the forest floor outside of each nest ($P = 0.11$). We also observed no differentiation in the composition of bacterial community membership between natural and supplemental structures (PERMANOVA: $P = 0.58$; Figure 2); however, we did detect differences between bacterial communities in Key Largo woodrat nests overall compared to the surrounding forest environment (PERMANOVA: $P < 0.001$; Figure 2).

Differences among bacterial communities in Key Largo woodrat nests (relative to the forest floor) were not driven by an accumulation of bacteria from pathogenic lineages. Their relative abundance was only slightly higher in nests than from the forest floor (percent of bacterial sequence reads: natural = 2.6%, supplemental = 3.1%, forest = 1.6%; $\chi^2 = 7.93$, $P = 0.005$), and there were no more of these pathogenic groups in supplemental structures than in

natural nests (χ^2 =1.801, P = 0.18). Of the 45 genera tested that contain rodent-associated pathogens, we detected only 12. These included *Bacillus*, *Burkholderia*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Eubacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptococcus*, and *Vibrio*, eight of which were shared between natural and supplemental nests (Table 1a). Furthermore, we found all recovered taxa at very low abundances (read numbers), with *Mycobacterium* accounting for the greatest relative abundance at 0.015% of total bacterial sequence reads in natural nest samples and 0.011% of bacterial sequences from supplemental nest samples (Table 1a). *Mycobacterium* was followed by *Burkholderia* (0.003% of bacterial sequences) in natural nests and *Nocardia* in supplemental nests (0.008% of bacteria sequences), of which only *Nocardia* was more abundant in nests than from forest floor samples (*Burkholderia*: χ^2 = 0.041, P = 0.84; *Nocardia*: χ^2 = 9.401, P = 0.002; Table 1a).

However, while we saw little accumulation of pathogenic taxa, we saw a high relative abundance of Pseudonocardiaceae and Streptomycetaceae in Key Largo woodrat nests. Both of these families are important antibiotic producing groups (Platas et al., 1998; Kämpfer et al., 2014) and include the bacteria that produce many of our common commercial antibiotics, such as erythromycin and vancomycin (a well-known treatment for Methicillin-resistant *Staphylococcus aureus* infections; Jafari et al., 2014; Kämpfer et al., 2014; Sakoulas et al., 2004). Relative to other taxa, Pseudonocardiaceae and Streptomycetaceae were the most abundant bacteria in both natural and supplemental nests, accounting for 12% of all sequence reads from natural nests and 15% of all sequence reads from supplemental nests. And though these taxa are found in diverse environments and are abundant in soils globally, they were notably more abundant in nests compared to the forest floor (only 4% of sequence reads from forest samples; χ^2 = 9.486, P = 0.002).

Due to the accumulation of Pseudonocardiaceae bacteria in nests, we also tested for its presence on rodent bodies. Just as with their nests, Pseudonocardiaceae was abundant on Key Largo woodrat bodies, where it was the third most abundant bacterial family collected from both the Key Largo woodrat (7% of bacterial sequences) and Key Largo cotton mouse (4.5% of bacterial sequences). We also found Pseudonocardiaceae on black rat individuals, albeit at lower abundances (2% of bacterial sequences). When we considered the accumulation of pathogenic taxa on rodent bodies, we observed 20 of the 45 genera considered (Key Largo woodrat = 16 genera, Key Largo cotton mice = 17 genera, and black rat = 17 genera; Table 1b). There was a slightly higher relative abundance of pathogenic taxa on bodies than in nests (percent of bacterial sequence reads: bodies = 6.1%, nests = 2.9%); however, this difference was not significant (χ^2 = 2.005, P = 0.157), and the magnitude of this trend was not consistent, when we considered each rodent species separately. The Key Largo woodrat had approximately the same relative abundance of pathogenic taxa (2.4% of bacterial sequences) as their natural nests (2.9% of bacterial sequences; P = 0.415). The black rat, however, had more than twice that of the Key Largo woodrat (5.5% of bacterial sequences), and the Key Largo cotton mouse had the greatest abundance overall. On average, 10.4% of the bacteria found on Key Largo cotton mice were from a lineage known to contain rodent-associated pathogens. More specifically, we found *Corynebacterium* to be the most abundant of these genera on Key Largo woodrats and black rats (0.006% and 0.021% of total bacterial sequences, respectively), and *Mycobacterium* was the most abundant taxa on Key Largo cotton mice (0.026% of bacterial sequences; Table 1b). *Mycobacterium* was found on all rodent species (as well as in nests) and includes noxious pathogens, such as those known to cause tuberculosis and leprosy in mammals (Koch, 1884; Gordon & Parish, 2018; Hansen, 1874).

When we considered all bacteria on bodies, regardless of origin, we observed more variation in bacterial communities on bodies than between those of nests and the forest floor (Figure 2). Forest floor samples showed the least amount of variation in community membership, followed by Key Largo woodrat nests (regardless of nest type), and though there was differentiation between nest and forest floor environments (PERMANOVA: $P < 0.001$), we found even more distinctive bacterial communities between the Key Largo woodrat, Key Largo cotton mouse, and black rat individuals (PERMANOVA: $P < 0.001$; Figure 2). Additionally, we found Key Largo woodrats to be most similar to their nests, relative to the other species (Figure 2).

Discussion

Overall, we found bacterial communities in Key Largo woodrat nests to be distinct from the forest floor, but we found no differences between natural and supplemental nests based on the diversity or composition of bacteria. There was little accumulation of bacteria from pathogenic lineages in nests or on Key Largo woodrat individuals. Instead, we found a high abundance of bacteria from antimicrobial producing groups. The other rodent species known to use these nests, albeit less frequently (i.e., the Key Largo cotton mouse and black rat), had a lower abundance of antimicrobial producing bacteria on their bodies and a higher abundance of bacteria from pathogenic taxa. In addition, bacterial communities in Key Largo woodrat nests were more similar to those on the Key Largo woodrats than they were to the bacteria from Key Largo cotton mice and black rats, suggesting that there is some interaction between Key Largo woodrat and nest microbiomes.

We expected supplemental nests to be similar to other structures built by humans (e.g., contemporary homes), in that, they might be expected to have a less diverse, unique assemblage

of bacteria compared to natural nests (Dunn et al., 2013; Lax et al., 2014). If supplemental nests alter bacterial species interactions, this could have detrimental effects on individual health. The exposure to a greater diversity of microbes increases immune response and the ability to fight off infectious disease in rodents (Beura et al., 2016). However, we found no evidence of such an effect. Relative to the forest floor, nests had similar bacterial diversity, regardless of whether they were natural or supplemental, and we could not tell the difference between nest type based on which bacteria were present. This suggests that, likely through some combination of nest design or pattern of use, supplemental nests maintain a bacterial community that is no different from their natural counterparts. One explanation might be that the culvert pipes used in supplemental nest construction have open ends. These openings could act in a similar way to the gaps in natural nests or to open windows in human homes (Kembel et al., 2012; Barberán et al., 2015).

We also found very little accumulation of bacteria from pathogenic lineages in natural and supplemental nests (Table 1a). With the exception of *Mycobacterium*, a genus that contains the bacteria that cause tuberculosis and leprosy (Koch, 1884; Gordon & Parish, 2018; Hansen, 1874) but which also includes a high diversity of harmless species, the most common pathogenic taxa were found at very low abundances (*Burkholderia* and *Nocardia*; less than 0.01% of total bacteria). *Burkholderia* and *Nocardia* are known to contain primarily non-pathogenic species, and further still, pathogens within *Nocardia* are frequently classified as opportunistic. High bacterial diversity and a high relative abundance of Pseudonocardiaceae and Streptomycetaceae in nests are likely contributing factors, as we know that higher bacterial diversity and the application of antimicrobials in human buildings are associated with a decrease in the relative abundance of pathogens (Lax et al., 2014; Ruokolainen et al., 2017). On the other hand, the application of antimicrobials in homes has favored antimicrobial-resistant strains (Hartmann et

al., 2016), and therefore, we may expect to find antibiotic-resistant bacteria in Key Largo woodrat nests, particularly since they are typically used for several generations and persist over long periods of time (Rainey, 1956). However, as our molecular methods are not reliable at the species level of identification (Martínez-Porchas et al., 2016; Edgar, 2018), it would be imprudent for us to make strong conclusions about the presence of individual species or strains within the scope of this study. Due to this, we cannot directly attribute the bacteria in Key Largo woodrat nests to pathogenesis but rather use this data as a proxy for understanding the broad-scale patterns of pathogen accumulation.

Since we know that the species found in human homes are influenced by the building's occupants (Barberán et al., 2015), we characterized the bacteria from the bodies of the rodents known to use Key Largo woodrat nests. Black rats had more than double the bacteria from pathogenic lineages as Key Largo woodrats. However, Key Largo cotton mice were host to the greatest abundance of pathogenic taxa, overall. Key Largo woodrats and black rats had a high abundance of *Corynebacterium*. Pathogens within this genus can cause disease, such as diphtheria and endocarditis, but it mostly contains non-pathogenic species and is a common associate of mammal skin (Loeffler, 1884; et Yersin, 1888; Almklov & Hansen, 1950; Pike, 1951). *Mycobacterium* was the most abundant pathogenic taxa on Key Largo cotton mice. However, aside from the presence of *Mycobacterium*, we primarily observed taxonomic groups that largely contain opportunistic rodent-associated pathogens. The low detection of pathogens in nests and on bodies may be due, in part, to the high abundances of Pseudonocardiaceae and Streptomycetaceae. Additionally, we found bacteria on Key Largo woodrats to be most similar to the bacteria in their nests, relative to the other species (Figure 2). The Key Largo cotton mouse and black rat do not inhabit nests as frequently or for long periods of time within the surveyed areas. It is also notable that the differentiation and variation among rodent microbiomes was

much greater than what we observed between nest and forest samples. This may be a phylogenetic artifact, since the Key Largo woodrat, Key Largo cotton mouse and black rat are all distantly related species (Steppan & Schenk, 2017). Another potential explanation could be the landfall of Hurricane Irma in September 2017 (3 months prior to sample collection). Catastrophic weather events can homogenize biological communities (Savage et al., 2018), and therefore, the hurricane could account for the similarity between and among nest and forest microbiomes.

One of our more unusual findings was the prevalence of bacteria from the Pseudonocardiaceae and Streptomycetaceae families in Key Largo woodrat nests. Associations between animals and antimicrobial producing bacteria have been described in social insects (e.g., from ants and wasps; Currie et al., 1999; Cafaro & Currie, 2005; Madden et al., 2013). However, to our knowledge, such a relationship has never been observed within mammals. Based on our study design, we cannot ascribe a causative relationship between the Key Largo woodrats and the presence of these bacteria. However, due to their high abundance and ubiquity among natural and supplemental nests, we propose the possibility that the bodies or behaviors of Key Largo woodrats promote the accumulation of these antimicrobial producing bacteria.

Of relevance to the management of Key Largo woodrats, supplemental nests do not appear to alter microbial species interactions in the ways we would predict to be detrimental to individual health.

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Tables and Figures

Table 1. Bacterial genera detected in our samples that contain known rodent-associated pathogens. All values represent the average percent relative abundance of total bacterial sequence reads per sample type. Taxa with the greatest relative abundance are marked with an asterisk (*). *(a)* Bacterial genera identified from natural nests, supplemental nests, and the forest floor. *(b)* Bacterial genera identified from the bodies of the native Key Largo (KL) woodrat, native Key Largo (KL) cotton mouse, and exotic black rat.

(a)

Bacterial genera	**Natural**	**Supplemental**	**Forest**
Bacillus	0.003	0.003	0.004*
Burkholderia	0.004*	0.001	7.00E-04
Clostridium	0	2.00E-05	0
Corynebacterium	4.00E-05	0.001	2.00E-05
Enterococcus	2.00E-05	0	2.00E-05
Eubacterium	0	0	2.00E-05
Micrococcus	0.0001	0.002	6.00E-05
Mycobacterium	0.0148	0.0112	1.46E-02
Nocardia	0.003	0.008*	2.00E-04
Rhodococcus	0.0002	0.001	0.0001
Streptococcus	0	0	4.00E-05
Vibrio	0.0004	0.0001	0

(b)

Bacterial genera	**KL woodrat**	**KL cotton mouse**	**Black rat**
Bacillus	0.002	0.002	0.0003
Burkholderia	4.00E-04	0.001	1.00E-04
Campylobacter	0	0	0.001
Clostridium	0.001	0.005	0.002
Corynebacterium	0.006*	0.01	0.021*
Enterococcus	0.002	0.001	0.004
Eubacterium	0	0.002	0.0003
Haemophilus	7.00E-04	0.002	4.00E-05
Helicobacter	0	0.009	0
Mannheimia	0.0003	0	2.00E-05
Micrococcus	0.0005	0.002	0.0003
Moraxella	2.00E-05	0	0.001
Mycobacterium	0.004	0.026*	0.006
Mycoplasma	2.00E-05	0.015	0.001
Nocardia	0.001	0.002	0.006
Rhodococcus	7.00E-04	0.016	3.00E-04
Stenotrophomonas	0.0001	0.001	0.001
Streptococcus	0.005	0.01	0.011
Treponema	0.001	0.002	0
Vibrio	0	0.0002	0

(a)

(b)

Figure 1. Natural and supplemental Key Largo woodrat nests in the tropical hardwood hammock forest of the Crocodile Lake National Wildlife Refuge. *(a)* Natural nests are built from downed wood and forest debris. The PVC pipe indicates the nest entrance that was sampled during this study. *(b)* Supplemental nests are built from large plastic culvert pipes surrounded and covered by rocks or chunks of fossilized coral.

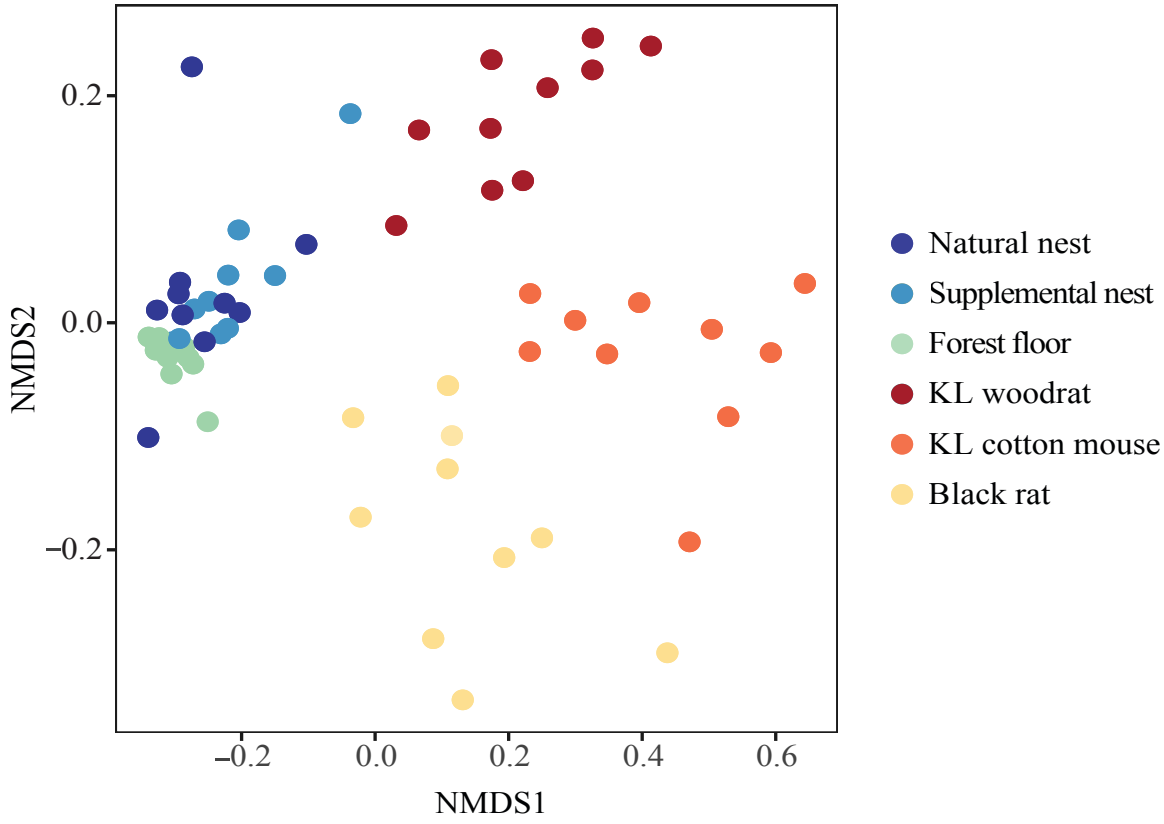

Figure 2. Non-metric multidimensional scaling (NMDS) ordination plot representing bacterial communities collected from natural and supplemental Key Largo (KL) woodrats nests and the forest floor outside each natural nest, as well as from the bodies of the rodent species that are known nest inhabitants (i.e., Key Largo woodrats, Key Largo cotton mice, and black rats). There were distinct bacterial communities within Key Largo woodrat nests compared to the forest floor ($P < 0.001$), but there was no difference between natural and supplemental structures ($P = 0.58$). However, there was strong differentiation of bacterial communities among rodent species ($P < 0.001$).

APPENDICES

Appendix A. Chapter 1 Supplemental Material

Table S1. Pairwise distances between 18S rDNA sequences from *Demodex* species. Lower left = Kimura 2-parameter distances; Upper right = number of nucleotide differences.

https://doi.org/10.1371/journal.pone.0106265.s001

		AY620908	JF834894	HQ728000	141: KF745881	176: KF745887	176: KF745886	315: KF745890	127: KF745876	315: KF745889	176: KF745888	GU377177	JN885463	JN885464	HQ727998	JN885468	KC010484	KC010485	JN885469	HQ718592	GU377178	JN885466	HQ727999	JN885465	315: KF745892	127: KF745878	127: KF745880	141: KF745884	141: KF745885	127: KF745877	141: KF745882	315: KF745891	127: KF745879	141: KF745883	JN885467	D.sp. (whitetailed d
Neochelacheles messersmithi	AY620908		48.00	48.00	48.00	48.00	48.00	48.00	48.00	48.00	49.00	48.00	47.00	48.00	50.00	51.00	50.00	50.00	50.00	52.00	52.00	51.00	51.00	51.00	51.00	51.00	51.00	51.00	51.00	51.00	52.00	53.00	53.00	49.00	49.00
D.musculi	JF834894	0.167		5.00	4.00	4.00	4.00	4.00	4.00	4.00	5.00	5.00	5.00	4.00	8.00	9.00	7.00	7.00	10.00	13.00	13.00	12.00	11.00	10.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	9.00	3.00
D.folliculorum	HQ728000	0.167	0.016		1.00	1.00	1.00	1.00	1.00	1.00	2.00	0.00	2.00	1.00	7.00	10.00	8.00	8.00	11.00	12.00	12.00	13.00	12.00	11.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	10.00	8.00
D.folliculorum	141: KF745881	0.167	0.013	0.003		0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	0.00	8.00	9.00	7.00	7.00	10.00	13.00	13.00	12.00	11.00	10.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	9.00	7.00
D.folliculorum	176: KF745887	0.167	0.013	0.003	0.000		0.00	0.00	0.00	0.00	1.00	1.00	1.00	0.00	8.00	9.00	7.00	7.00	10.00	13.00	13.00	12.00	11.00	10.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	9.00	7.00
D.folliculorum	176: KF745886	0.167	0.013	0.003	0.000	0.000		0.00	0.00	0.00	1.00	1.00	1.00	0.00	8.00	9.00	7.00	7.00	10.00	13.00	13.00	12.00	11.00	10.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	9.00	7.00
D.folliculorum	315: KF745890	0.167	0.013	0.003	0.000	0.000	0.000			0.00	1.00	1.00	1.00	0.00	8.00	9.00	7.00	7.00	10.00	13.00	13.00	12.00	11.00	10.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	9.00	7.00
D.folliculorum	127: KF745876	0.167	0.013	0.003	0.000	0.000	0.000	0.000			0.00	1.00	1.00	1.00	0.00	8.00	9.00	7.00	7.00	10.00	13.00	13.00	12.00	11.00	10.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	9.00	7.00
D.folliculorum	315: KF745889	0.167	0.013	0.003	0.000	0.000	0.000	0.000	0.000		1.00	1.00	1.00	0.00	8.00	9.00	7.00	7.00	10.00	13.00	13.00	12.00	11.00	10.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	9.00	7.00
D.folliculorum	176: KF745888	0.171	0.016	0.006	0.003	0.003	0.003	0.003	0.003	0.003		2.00	2.00	1.00	9.00	10.00	8.00	8.00	9.00	12.00	12.00	11.00	10.00	9.00	13.00	14.00	13.00	13.00	13.00	13.00	13.00	15.00	15.00	15.00	8.00	8.00
D.folliculorum	GU377177	0.167	0.016	0.000	0.003	0.003	0.003	0.003	0.003	0.003	0.006		2.00	1.00	7.00	10.00	8.00	8.00	11.00	12.00	12.00	13.00	12.00	11.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	10.00	8.00
D.folliculorum	JN885463	0.163	0.016	0.006	0.003	0.003	0.003	0.003	0.003	0.003	0.006	0.006		1.00	9.00	10.00	8.00	8.00	11.00	14.00	14.00	13.00	12.00	11.00	15.00	16.00	15.00	15.00	15.00	15.00	15.00	16.00	17.00	17.00	10.00	8.00
D.folliculorum	JN885464	0.167	0.013	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.003	0.003		8.00	9.00	7.00	7.00	10.00	13.00	13.00	12.00	11.00	10.00	14.00	15.00	14.00	14.00	14.00	14.00	14.00	15.00	16.00	16.00	9.00	7.00
D.canis	HQ727998	0.175	0.025	0.022	0.025	0.025	0.025	0.025	0.025	0.025	0.028	0.022	0.029	0.025		3.00	1.00	1.00	15.00	16.00	16.00	17.00	16.00	15.00	18.00	19.00	18.00	18.00	18.00	18.00	18.00	19.00	20.00	20.00	13.00	11.00
D.canis	JN885468	0.178	0.028	0.032	0.028	0.028	0.028	0.028	0.028	0.028	0.032	0.032	0.032	0.028	0.009		2.00	2.00	12.00	15.00	15.00	14.00	13.00	12.00	16.00	17.00	16.00	16.00	16.00	16.00	16.00	17.00	18.00	18.00	12.00	12.00
D.canis	KC010484	0.175	0.022	0.025	0.022	0.022	0.022	0.022	0.022	0.022	0.025	0.025	0.025	0.022	0.003	0.006		0.00	14.00	17.00	17.00	16.00	15.00	14.00	18.00	19.00	18.00	18.00	18.00	18.00	18.00	19.00	20.00	20.00	12.00	10.00
D.canis	KC010485	0.175	0.022	0.025	0.022	0.022	0.022	0.022	0.022	0.022	0.025	0.025	0.025	0.022	0.003	0.006	0.000		14.00	17.00	17.00	16.00	15.00	14.00	18.00	19.00	18.00	18.00	18.00	18.00	18.00	19.00	20.00	20.00	12.00	10.00
D.canis	JN885469	0.174	0.032	0.035	0.032	0.032	0.032	0.032	0.032	0.032	0.028	0.035	0.035	0.032	0.048	0.038	0.045	0.045		3.00	3.00	2.00	3.00	2.00	6.00	5.00	4.00	4.00	4.00	6.00	6.00	8.00	6.00	6.00	2.00	13.00
D.brevis	HQ718592	0.182	0.042	0.038	0.042	0.042	0.042	0.042	0.042	0.042	0.038	0.038	0.045	0.042	0.051	0.048	0.055	0.055	0.009		0.00	5.00	6.00	3.00	8.00	7.00	6.00	6.00	6.00	8.00	8.00	10.00	8.00	8.00	5.00	16.00
D.brevis	GU377178	0.182	0.042	0.038	0.042	0.042	0.042	0.042	0.042	0.042	0.038	0.038	0.045	0.042	0.051	0.048	0.055	0.055	0.009	0.000		5.00	6.00	3.00	8.00	7.00	6.00	6.00	6.00	8.00	8.00	10.00	8.00	8.00	5.00	16.00
D.brevis	JN885466	0.178	0.038	0.042	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.035	0.042	0.042	0.038	0.055	0.045	0.051	0.051	0.006	0.016	0.016		3.00	4.00	6.00	5.00	6.00	6.00	6.00	6.00	8.00	8.00	8.00	4.00	15.00
D.brevis	HQ727999	0.178	0.035	0.038	0.035	0.035	0.035	0.035	0.035	0.035	0.032	0.038	0.038	0.035	0.052	0.042	0.048	0.048	0.009	0.019	0.019	0.009		3.00	3.00	4.00	5.00	5.00	5.00	3.00	3.00	5.00	7.00	7.00	5.00	14.00
D.brevis	JN885465	0.178	0.032	0.035	0.032	0.032	0.032	0.032	0.032	0.032	0.029	0.035	0.035	0.032	0.048	0.038	0.045	0.045	0.006	0.009	0.009	0.009	0.013	0.009		6.00	7.00	6.00	6.00	6.00	6.00	8.00	8.00	8.00	4.00	13.00
D.brevis	315: KF745892	0.179	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.042	0.045	0.048	0.045	0.058	0.052	0.058	0.058	0.019	0.025	0.025	0.019	0.009	0.019		1.00	2.00	2.00	2.00	0.00	0.00	2.00	4.00	4.00	8.00	15.00
D.brevis	127: KF745878	0.179	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.045	0.048	0.051	0.048	0.062	0.055	0.062	0.062	0.016	0.022	0.022	0.016	0.013	0.022	0.003		1.00	1.00	1.00	1.00	1.00	3.00	3.00	3.00	7.00	16.00
D.brevis	127: KF745880	0.179	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.042	0.045	0.048	0.045	0.058	0.051	0.058	0.058	0.013	0.019	0.019	0.019	0.016	0.019	0.006	0.003		0.00	0.00	2.00	2.00	4.00	2.00	2.00	6.00	15.00
D.brevis	141: KF745884	0.179	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.042	0.045	0.048	0.045	0.058	0.051	0.058	0.058	0.013	0.019	0.019	0.019	0.016	0.019	0.006	0.003	0.000		0.00	2.00	2.00	4.00	2.00	2.00	6.00	15.00
D.brevis	141: KF745885	0.179	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.042	0.045	0.048	0.045	0.058	0.051	0.058	0.058	0.013	0.019	0.019	0.019	0.016	0.019	0.006	0.003	0.000	0.000		2.00	2.00	4.00	2.00	2.00	6.00	15.00
D.brevis	127: KF745877	0.179	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.042	0.045	0.048	0.045	0.058	0.052	0.058	0.058	0.019	0.025	0.025	0.019	0.009	0.019	0.000	0.003	0.006	0.006	0.006		0.00	2.00	4.00	4.00	8.00	15.00
D.brevis	141: KF745882	0.179	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.042	0.045	0.048	0.045	0.058	0.052	0.058	0.058	0.019	0.025	0.025	0.019	0.009	0.019	0.000	0.003	0.006	0.006	0.000		2.00	4.00	4.00	2.00	6.00	15.00
D.brevis	315: KF745891	0.183	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.052	0.048	0.062	0.055	0.062	0.062	0.025	0.032	0.032	0.025	0.016	0.025	0.006	0.009	0.013	0.013	0.013	0.006	0.006		6.00	6.00	10.00	16.00
D.brevis	127: KF745879	0.187	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.048	0.051	0.055	0.051	0.065	0.058	0.065	0.065	0.019	0.025	0.025	0.025	0.022	0.025	0.013	0.009	0.006	0.006	0.006	0.013	0.013	0.019		0.00	8.00	17.00
D.brevis	141: KF745883	0.187	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.048	0.051	0.055	0.051	0.065	0.058	0.065	0.065	0.019	0.025	0.025	0.025	0.022	0.025	0.013	0.009	0.006	0.006	0.006	0.013	0.013	0.019	0.000		8.00	17.00
D.canis	JN885467	0.170	0.028	0.032	0.028	0.028	0.028	0.028	0.028	0.028	0.025	0.032	0.032	0.028	0.042	0.038	0.038	0.038	0.006	0.016	0.016	0.013	0.016	0.013	0.025	0.022	0.019	0.019	0.019	0.025	0.025	0.032	0.025	0.025		12.00
D.sp. (whitetailed deer)	KC010483	0.171	0.009	0.025	0.022	0.022	0.022	0.022	0.022	0.022	0.025	0.025	0.025	0.022	0.035	0.038	0.032	0.032	0.042	0.051	0.051	0.048	0.045	0.042	0.048	0.051	0.048	0.048	0.048	0.048	0.048	0.051	0.055	0.055	0.038	

Material S1. *Demodex* 18S rDNA sequence alignment. (FAS)

https://doi.org/10.1371/journal.pone.0106265.s002

Material S2. Tree file for the maximum likelihood (ML) tree. (TRE)

https://doi.org/10.1371/journal.pone.0106265.s003

Material S3. Tree file for the Bayesian inference (BI) tree. (TRE)

https://doi.org/10.1371/journal.pone.0106265.s004

Appendix B. Chapter 2 Supplemental Material

Table S1. OTUs removed prior to rarefication and analyses. All removed OTUs were spurious, of low quality, or designated as unassigned, mitochondrial, or chloroplast sequences. To provide support for the removal of any OTUs with greater than 1000 reads across all samples, NCBI blast results were reported.

Access here: http://rsos.royalsocietypublishing.org/content/5/5/180382.figures-only

Table S2. Body associates. Chimpanzee body-associated bacterial taxa used in source-tracking analyses.

Fecal	**Oral**
Ruminococcus	*Enterobacter*
Roseburia	*Serratia*
Oscillabacter	*Escherichia*
Anaerovorax	*Citrobacter*
Novosphingobium	*Salmonella*
Caprococcus	*Klebsiella*
Parabacteroides	*Buttiauxella*
Blautia	*Kluyvera*
Faecalibacterium	*Providencia*
Subdoligranulum	*Bibersteinia*
Anaerotruncus	*Actinobacillus*
Anaeroplasma	*Haemophilus*
Dorea	*Pasteurella*
Prevotella	*Aggregatibacter*
Oribacterium	*Neisseria*
Bulleidia	*Streptococcus*
Lachnospiraceae	*Granulicatella*
	Veillonella
Skin	*Rothia*
Corynebacterium	*Actinomyces*
Staphylococcaceae	*Fusobacterium*
Anaerococcus	*Leptotrichia*
Prevotella	*Streptobacillus*
Arthrobacter	*Chryseobacterium*
Sphingomonas	*Capnocytophaga*
Microbacteriaceae	*Prevotella*
Agrobacterium	*Porphyromonas*
Phycicoccus	
M. adhaesivum	

Table S3. PERMANOVA results. Data were analyzed following rarefication. All potential explanatory variables were included within both the OTU richness and community composition PERMANOVA models, using an FDR correction for multiple comparisons. Variables included whether a sample was collected from a chimpanzee bed, the age of a bed, season (wet or dry), elevation above sea level (m), and whether a sample was from a branch or a leaf. (a) Alpha richness values were calculated based on the number of unique microbial OTUs present in each sample. (b) Community composition data were weighted by OTU abundance using the Bray-Curtis dissimilarity metric.

(a) Diversity						
	Full Model		Bed		Environment	
All	R^2	*P*	R^2	*P*	R^2	*P*
Bed vs. Environment	0.002	0.509	.	.	.	.
Season	0.432	0.001	0.543	0.001	0.321	0.001
Elevation	0.312	0.011	0.336	0.559	0.492	0.381
Tree Surface	0.004	0.402	0.001	0.825	0.011	0.407
Bed Age	.	.	0.004	0.625	.	.
(b) Community Composition						
	Full Model		Bed		Environment	
All	R^2	*P*	R^2	*P*	R^2	*P*
Bed vs. Environment	0.026	0.001	.	.	.	.
Season	0.139	0.001	0.201	0.001	0.116	0.001
Elevation	0.463	0.001	0.638	0.002	0.666	0.001
Tree Surface	0.023	0.003	0.025	0.043	0.041	0.004
Bed Age	.	.	0.01	0.714	.	.

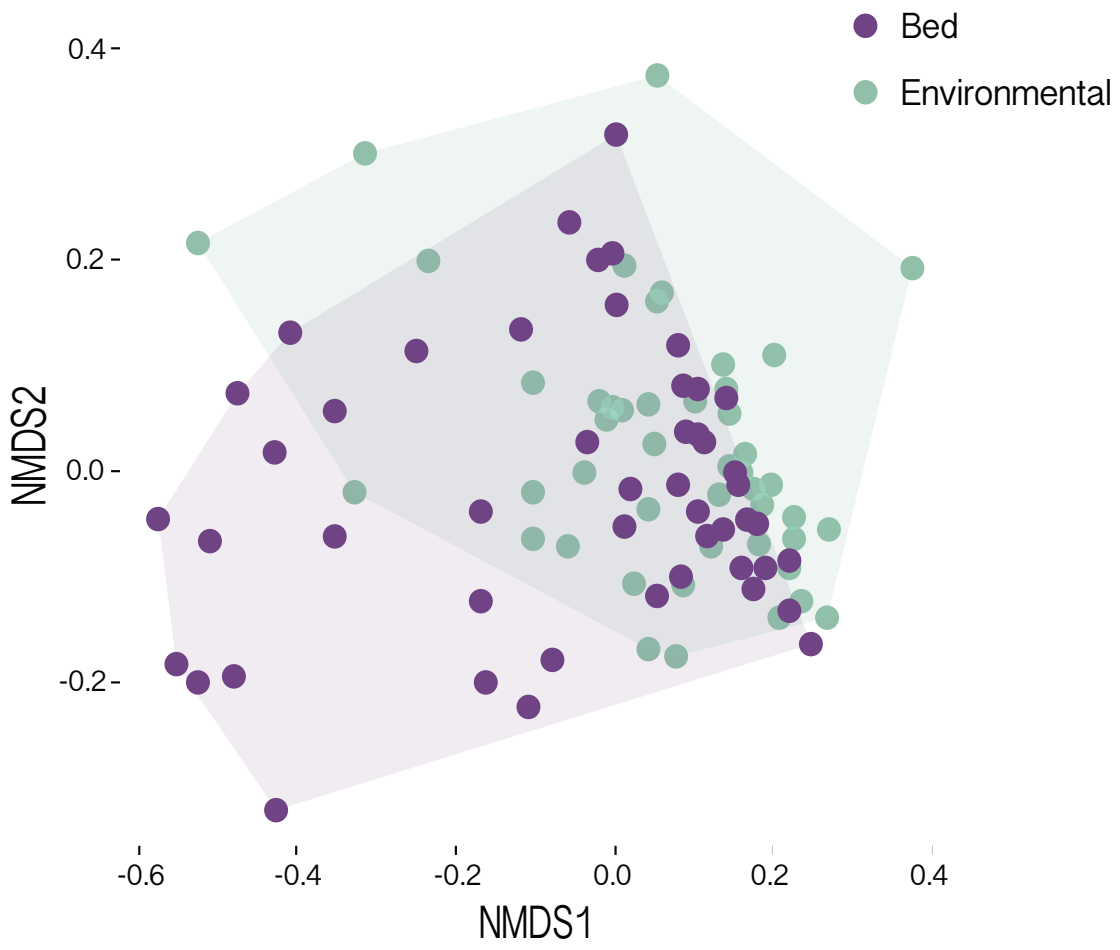

Figure S1. Nonmetric multidimensional scaling (NMDS) ordination plot. NMDS plot representing the overall variation in microbial community composition as a function of sample type. Sites are coded based on whether they were collected from within a chimpanzee bed or from environmental samples (leaves and branches of the same tree). Note a differentiation between a subset of beds (bottom left) and the environment ($p < 0.001$).

Appendix C. Chapter 3 Supplemental Material

Table S1. Data from previous studies combined with our data (i.e., from Namibian and United States homes) to compare bacterial communities from modern homes to those from a range of built environments. These studies included bacteria collected from chimpanzee nests, additional homes in the United States, and the International Space Station.

Study	Publication	16S variable region	Number of samples
Chimpanzee nests	Thoemmes et al. 2018	v1-v2	48
Namibian homes	NA	v4-v5	10
United States homes	Dunn et al. 2013	v4-v5	120
International Space Station	Lang et al. 2018	v4-v5	15

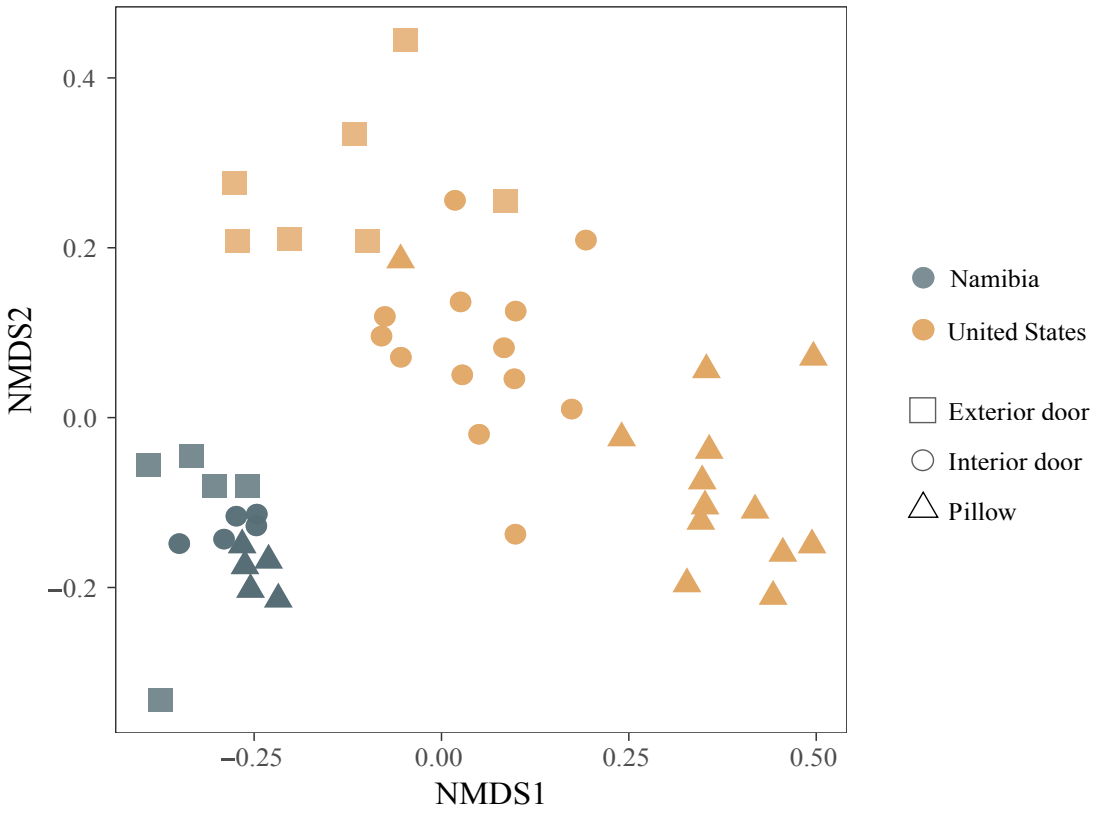

Figure S1. Non-metric multidimensional scaling (NMDS) ordination plot of bacterial communities from Namibian and United States homes by sample habitat. There was no differentiation of habitats in Namibian homes ($P = 0.183$); however, there were distinct bacterial communities among habitats for homes in the United States ($P < 0.001$).

Appendix D. Chapter 4 Supplemental Material

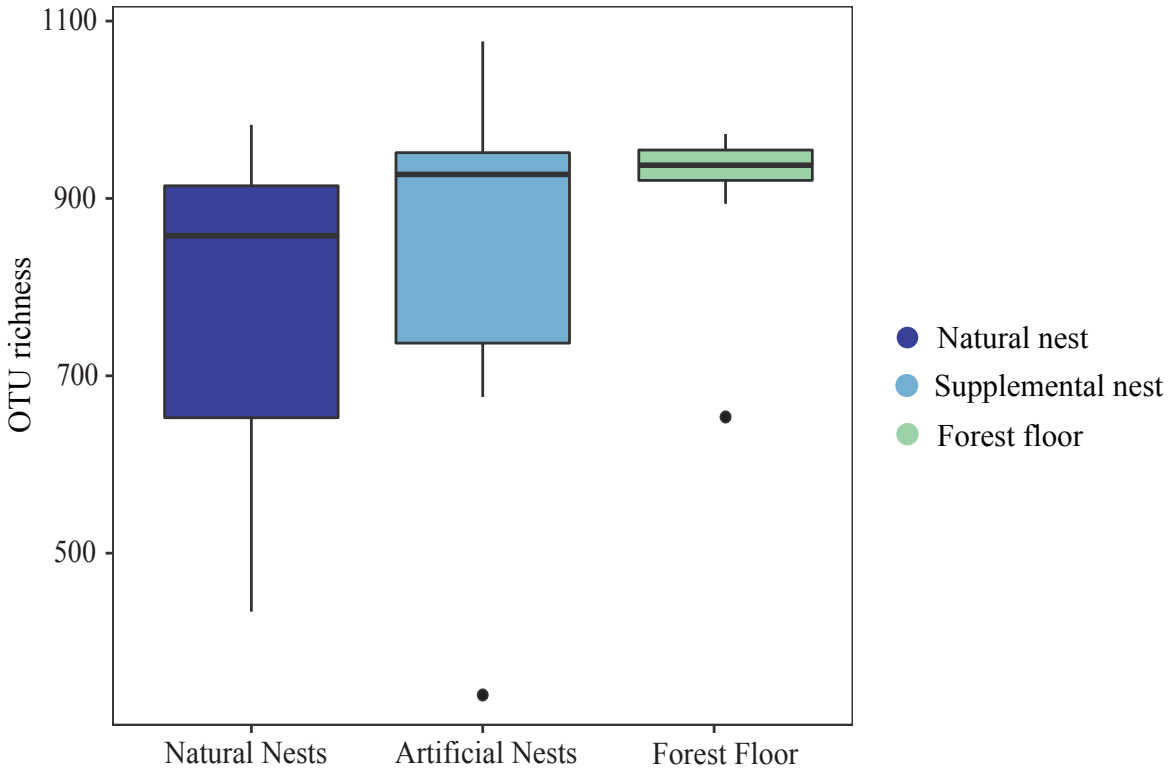

Figure S1. OTU richness of bacteria collected from natural nests, supplemental nests, and the forest floor outside of each natural nest for the Key Largo woodrat. There were no observed differences in OTU richness between sample type (natural and supplemental nests: $P = 0.41$; nests and forest floor: $P = 0.15$).

Appendix E. Supplemental Chapter 1

Global divergence of the human follicle mite *Demodex folliculorum*: Persistent associations between host ancestry and mite lineages

Palopoli M, Fergus DJ, Minot S, Pei D, Simison B, Fernandez-Silva I, Thoemmes MS, Dunn RR, & Trautwein M (2015) Global divergence of the human follicle mite *Demodex folliculorum*: Persistent associations between host ancestry and mite lineages. *PNAS* 112(52): 15958–15963.

Global divergence of the human follicle mite *Demodex folliculorum*: Persistent associations between host ancestry and mite lineages

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Microscopic mites of the genus *Demodex* live within the hair follicles of mammals and are ubiquitous symbionts of humans, but little molecular work has been done to understand their genetic diversity or transmission. Here we sampled mite DNA from 70 human hosts of diverse geographic ancestries and analyzed 241 sequences from the mitochondrial genome of the species *Demodex folliculorum*. Phylogenetic analyses recovered multiple deep lineages including a globally distributed lineage common among hosts of European ancestry and three lineages that primarily include hosts of Asian, African, and Latin American ancestry. To a great extent, the ancestral geography of hosts predicted the lineages of mites found on them; 27% of the total molecular variance segregated according to the regional ancestries of hosts. We found that *D. folliculorum* populations are stable on an individual over the course of years and that some Asian and African American hosts maintain specific mite lineages over the course of years or generations outside their geographic region of birth or ancestry. *D. folliculorum* haplotypes were much more likely to be shared within families and between spouses than between unrelated individuals, indicating that transmission requires close contact. Dating analyses indicated that *D. folliculorum* origins may predate modern humans. Overall, *D. folliculorum* evolution reflects ancient human population divergences, is consistent with an out-of-Africa dispersal hypothesis, and presents an excellent model system for further understanding the history of human movement.

Demodex | phylogeography | symbiosis | coevolution

Human evolution did not take place in isolation but instead occurred alongside that of many closely associated species. Phylogeographic studies of human-associated species—such as lice and rodents, as well as certain bacteria and viruses—have suggested, eliminated, and confirmed hypotheses about human history (1–10). For example, these studies have provided details about the timing and nature of the original human migration out of Africa, the spread of humans within and among continents, and the domestication of large vertebrates.

Mites of the genus *Demodex* live in the hair follicles and sebaceous glands of humans and provide a promising system with which to explore further the details of human evolution. The association between *Demodex* and *Homo sapiens* is likely to be an ancient one: The broad distribution of these mites across mammal species (11), coupled with the ancient date of divergence estimated between the two species known to be found on humans (12), suggests that *Demodex* originated and diversified with early mammals. Furthermore, *Demodex* seem likely to have been carried along whenever their hosts migrated, because they are ubiquitous inhabitants of human skin (13, 14). Finally, in comparison with the other human associates that have been studied to date, *Demodex* mites are more tightly associated with human bodies than are lice, while their generation times are slower than those of bacteria and viruses but are faster than those of rodents, making them a complementary system with which to understand the evolution of both humans and human associates.

Two species of *Demodex* are known to inhabit the skin of humans. Histological studies suggest that each occupies a different niche: *Demodex folliculorum* resides in the hair follicle and is often found near the skin surface, whereas *Demodex brevis* is generally found deep in the sebaceous glands (15). As a result, the frequency of *D. folliculorum* movement from one host to another may be greater than that of *D. brevis*. A recent phylogenetic analysis of *Demodex*, including the two human associates, shows geographically structured genetic variation in *D. brevis* in which individuals of European descent and those of temperate Asian (Chinese) descent exhibit up to 6% divergence in nuclear ribosomal 18S sequence (14). In contrast, studies based on 18S rDNA and 16S mtDNA suggest that *D. folliculorum* exhibits no clear geographic structure among hosts from China, Spain, Brazil, and the United States (14, 16, 17). However, without additional sampling it is impossible to know whether the absence of apparent geographic structure in *D. folliculorum* truly reflects high rates of

Significance

Mites live in human hair follicles and have been implicated in medically important skin disorders, but we know surprisingly little about these residents of our skin. By analyzing the variation segregating among 241 mite sequences isolated from 70 human hosts, we showed that hosts with different regional ancestries harbor distinct lineages of mites and that these associations can persist despite generations spent in a new geographic region. These results suggest that some mite populations are better able to survive and reproduce on hosts from certain geographic regions. Improving our understanding of human follicle mites promises to shed light on human evolution and to provide important contextual information for their role in human health.

Author contributions: M.F.P., D.J.F., S.M., R.R.D., and M.T. designed research; M.F.P., D.J.F., S.M., D.T.P., M.S.T., and M.T. performed research; M.F.P., D.J.F., S.M., W.B.S., I.F.-S., and M.T. analyzed data; and M.F.P., R.R.D., and M.T. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession nos. KU174704–KU174944).

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global gene flow or instead is an artifact of limited global sampling and the particular genetic loci studied.

Key to understanding the global phylogeography of these mites is an understanding of how they move among hosts. The transfer of mites from mother to progeny and between mating partners has been demonstrated in nonhuman mammals (18–21). However, the movement of *Demodex* among human hosts has not been characterized. If human mites are transferred between hosts at high rates, the resulting high rates of migration could account for the limited geographic structure observed in *D. folliculorum* to date.

Here we used a 930-bp fragment of the mitochondrial genome to evaluate the genetic diversity and phylogeography of *D. folliculorum* among 70 human hosts of diverse geographic origins and ancestries. Our samples included people of European, Asian, African, and Latin American descent, the majority of whom currently live in the United States, providing the most broadly sampled evolutionary tree to date for any *Demodex* species.

Additionally, we investigated *Demodex* transmission among humans in two ways. First, we sampled multiple mites from a single host individual over the course of 3 y to characterize the diversity and stability of the mite population. Second, we examined the relationships among mites on three sets of parents and their adult progeny; because of the close association among family members, we hypothesized that mite lineages are more likely to be shared within families than between unrelated hosts.

The study of *Demodex* mites speaks to the story of human evolution as well as the coevolution between symbiont and host. Moreover, understanding these mites and their microbes will have applied value, because they have been linked to skin disorders such as rosacea and blepharitis (22, 23). Whatever the influence of mites on these disorders may be, it may depend on the mite lineages inhabiting a particular host. Ultimately, elucidating the evolution and transmission of *Demodex* mites not only will be a useful step toward understanding the evolutionary history of humans but also will be critical to contextualizing their role in human health.

Results and Discussion

Analysis of variation in *D. folliculorum* mtDNA (241 sequences, 883 bp of overlap), based on mites isolated from 70 human hosts with diverse regional ancestries, revealed high genetic diversity,

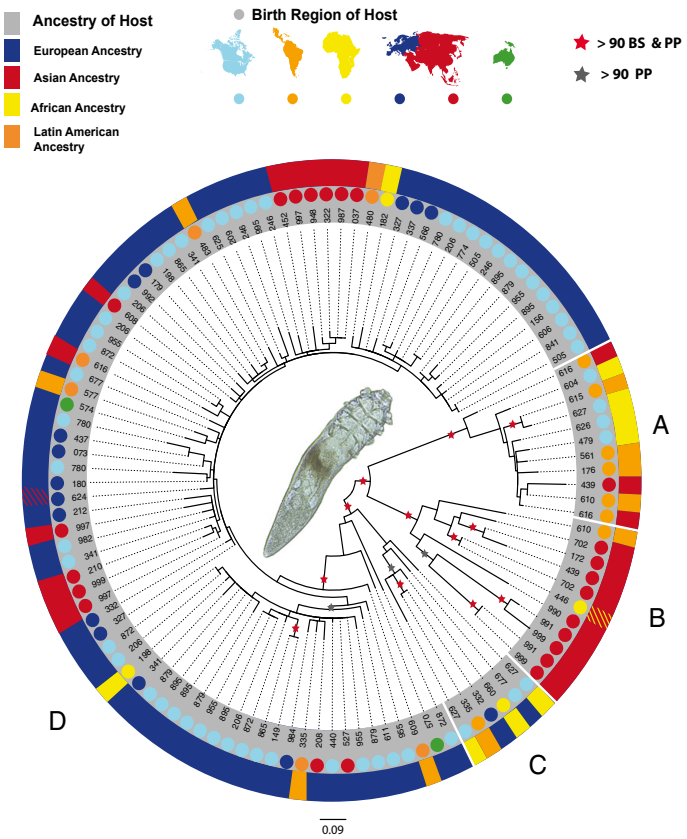

Fig. 1. Maximum likelihood (ML) tree of *D. folliculorum* mtDNA (883 bp, 70 hosts, 241 sequences). Dots indicate the continent on which a host was born (note that Latin American regions Mexico and Central America are grouped with South America). Colored rectangles above each dot indicate the host's continental ancestry. Rectangles of mixed colors indicate mixed parental ancestry. Red stars indicate bootstrap (BS) values and posterior probabilities (PP) are >0.90 from both ML and Bayesian analyses. Gray stars indicate nodes where only Bayesian posterior probabilities are >0.90. Multiple sequences from a single host that were either identical or clustered together in a single clade were collapsed into a single tree tip. See Figs. S1–S3 for alternative representations of this phylogeny. We recovered four major clades that differ in relative frequency depending on the geographic origins of the hosts. The great majority of hosts with European ancestry are included in clade D; clades A, B, and C primarily include hosts of African, Asian, and Latin American ancestry. A light micrograph of a *D. folliculorum* female is shown in the center.

Table 1. Molecular diversity indices for *D. folliculorum* mtDNA sequences grouped by the regional ancestry of their human hosts

Host regional ancestry	*n*	N_h	*h*	π	Fu's F_s	*P*	Harpending's *r*	*P*
European	158	71	0.96	0.02	**−24.32**	**<0.001**	0.014	0.131
African	17	9	0.83	0.05	4.84	0.97	**0.129**	**<0.001**
Asian	47	29	0.96	0.06	−1.07	0.40	**0.017**	**0.017**
Latin American	10	10	1.00	0.06	−1.47	0.13	0.058	0.413
Total	232	119	0.98	0.03				

Number of *D. folliculorum* specimens (*n*), number of haplotypes (N_h), haplotype diversity (*h*), and nucleotide diversity (π) are listed. Fu's F_S statistic and Harpending's *r* index are also listed. Significant values are indicated in bold. Nine sequences with missing data were excluded from these statistical analyses.

with four deeply divergent lineages evident in the global phylogeny (Fig. 1; see Figs. S1–S3 for alternative depictions of this phylogeny). When mites were grouped according to host regional ancestry, estimates of both haplotype and nucleotide diversity within each group were high (Table 1). Because we would not expect such high levels of diversity to be present if these mites had colonized humans only recently, these results support the hypothesis that *D. folliculorum* has had a long association with humans. Furthermore, these results suggest that *D. folliculorum* has not been through a severe population bottleneck in the recent past, despite evidence for a recent bottleneck among human populations (e.g., ref. 24).

Like other species that have an ancient association with humans (1, 6, 25), the evolutionary history of *D. folliculorum* appears to reflect historical patterns of human population divergences. First, a substantial proportion of the molecular variation segregated according to the regional ancestry of the hosts, as can be seen by comparing the frequencies of the four highly divergent clades (A, B, C, and D) among hosts with different regional ancestries (Fig. 2). Hosts with European ancestry almost exclusively hosted mites from clade D and lacked mites from clades A and B; in contrast, mites from clades A and B were relatively common on hosts with ancestry from Africa, Asia, or Latin America. Overall, ∼27% of the sequence variation in the mtDNA segregated according to host regional ancestry. Analysis of molecular variance (AMOVA) shows that such segregation was extremely unlikely to have occurred by chance ($\Phi_{ST} = 0.267$, $P < 0.000001$) (Table S1). Second, the observed patterns of mite diversity are consistent with an out-of-Africa model of human migration. As predicted by this model, the hosts of African descent harbored a very diverse sample of mite haplotypes, with all four divergent clades represented on only seven sampled hosts (18 sequences). Only a subset of this variation was present on hosts of either Asian or European descent: The former lacked mites from clade C, and the latter lacked mites from clades A and B, as would be expected if only a subset of this variation left Africa during human migrations.

One complexity that is not well accounted for by the out-of-Africa diversity model is that hosts from Latin America harbored a broad diversity of mites from all four divergent clades (Fig. 2). However, understanding the origins of *D. folliculorum* on hosts of Latin American ancestry is complicated by the many recent migrations of people into this region, resulting in a population of mixed African, European, and Native American ancestry (26, 27). Thus, *D. folliculorum* in Latin America may be from lineages that were endemic to African, European, or Native American hosts. When considering the ancestral make-up of Latin America, it also is noteworthy that over 10 times more Africans were brought to Brazil than to mainland North America during the slave trade from 1501 to 1866 (TransAtlantic Slave Trade Database; www.slavevoyages.org/; and ref. 26), so that there was a much larger source of African populations of *Demodex* in South America. These demographic patterns could explain why such great diversity was represented among mites from only eight Latin American hosts (12 sequences).

Three lines of evidence indicate that mite lineages remain stable on human hosts for long periods of time. First, mite populations appeared to be stable on an individual host over a 3-y period. A single individual of European descent (host 206) was sampled 36 times over the course of 3 y (2007–2009). Among the 36 mites collected from host 206, we found seven haplotypes that clustered into three haplogroups within clade D (Fig. S3). The same clade D haplotypes were recovered consistently from host 206 each year. An AMOVA on these sequences provided no evidence that molecular variation segregated according to year of mite isolation ($\Phi_{ST} = 0$, $P = 0.49$) (Table S1). These results are consistent with

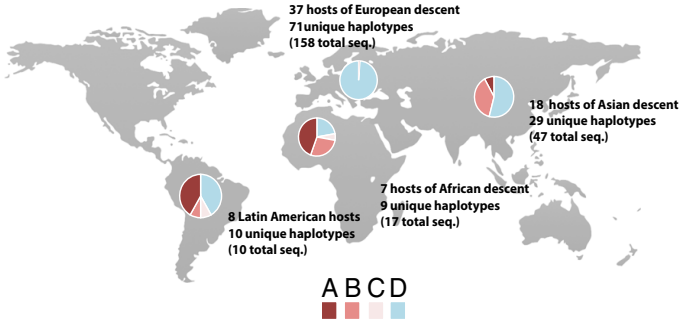

Fig. 2. Frequency of clade recovery according to the geographic region of host ancestry. Clades A, B, C, and D were recovered from African and Latin American hosts; Asian participants hosted only clades A, B, and D; Europeans primarily hosted mites from clade D. Sequences with missing data were excluded from host and haplotype counts.

the hypothesis that specific populations of *D. folliculorum* can persist on an individual host for years.

Second, hosts appeared to retain mite populations for years after moving to new geographic regions. Specifically, some hosts that were born in Asia and subsequently moved to the United States years before sampling nevertheless carried mites from clade B, which was common among mites sampled from Asian hosts but absent among mites sampled from hosts of European descent (Fig. 2). For example, host 702 was born in Asia but lived in the United States for 8 y before sampling occurred and during this time surely came into frequent contact with individuals of European descent; nevertheless, both mites isolated from this individual fell within clade B, suggesting that this host has retained distinctly Asian mites for 8 y.

Third, and perhaps most interestingly, hosts appeared to retain specific mite lineages for generations after moving to new geographic regions. We observed several examples of African American participants (hosts) whose ancestors have lived in the United States for multiple generations, but they carried mites from clade A, which was isolated only from hosts of African, Asian, and Latin American ancestry (Fig. 2). Certainly these hosts, and their ancestors, came into frequent contact with individuals of European descent. Given the apparent absence of clade A among individuals of European descent, our data suggest that these African American hosts have retained mite lineages originally inherited from Africa rather than having exchanged mite populations regularly with individuals of European descent.

One hypothesis to explain the persistence of *D. folliculorum* populations on hosts across years and even across generations is that the mites have extremely low dispersal rates; however, this hypothesis does not explain the observation that individual hosts often harbor diverse populations of mites (Fig. S3). An alternative hypothesis, which is perhaps more likely, is that hosts differ in the characteristics of their hair follicles and sebaceous glands, leading to differential fitness of some mite clades relative to others. In this "skin traits" model, the persistence of mite populations on particular hosts is the result of differential survival or reproduction rather than colonization. Human populations do differ in skin hydration, hair follicle density and morphology, and lipid production and composition (28, 29). Which of these skin attributes is most important to mite fitness is unknown.

The geographically widespread clade D exhibited less phylogenetic resolution and lower average levels of genetic diversity than the other clades (Fig. 1). Previous results, showing low 18S rDNA sequence divergence among *D. folliculorum* collected from hosts in China and in the Americas (14), are consistent with at least one globally widespread clade of *D. folliculorum*, potentially represented by clade D here (Fig. 2). The limited phylogenetic resolution and star-like structure of this clade indicates a recent (and sudden) expansion in the population from which these mites were sampled. This inference is supported by the negative and significant Fu's *Fs* test and a nonsignificant raggedness index (Harpending's *r*) observed among mites isolated from European hosts, which almost exclusively harbor mites from clade D (Table 1). These patterns persist even when samples from hosts of European ancestry are limited to exclude hosts from the same family and to include only one mite per host ($F_s = -15.97$, $P = 0.00004$; $r = 0.008$, $P = 0.595$), so they are unlikely to be an artifact of sampling bias. Indeed, closely related mites from clade D were recovered from hosts of diverse ancestries and from many regions around the world, including Nepal, Australia, Morocco, Peru, and the United States.

A basic question is whether the presence of closely related mites from clade D on a wide diversity of people reflects the ancient distribution of this lineage (with a lack of apparent geographic structure) or occurred more recently with the movement of humans around the globe. One interesting possibility is that this pattern may be the result of rapidly changing human distributions over the last few hundred years; in particular, Europeans may have spread clade D as they colonized many parts of the world. Mites from clade D also may have less specialized requirements for skin microhabitat than exhibited by mites from other clades. According to this hypothesis, the widespread distribution and rapid spread of clade D is facilitated by its higher rates of survival or reproduction on the hosts it colonizes.

D. folliculorum colonization also was studied by collecting samples of mites from three family groups of European descent; in each case, mother, father, and adult offspring were sampled. Mite haplotypes often were shared among members of the same family (Fig. S3). Mites sampled from the parents of host 206 (mother 895, father 872) clustered within the same clades as the mites sampled from their offspring and in some cases parents and offspring shared identical haplotypes. Similarly, haplotypes were shared within the other two family units sampled (offspring/mother/father: 955/879/677 and 841/505/246). Of the nine family members sampled, seven shared haplotypes with other family members. In contrast, we recovered relatively few haplotypes shared outside of family units. Of the 61 unrelated individuals sampled, only 13 shared haplotypes. The sharing of haplotypes by the mother and father and by the parents and their offspring was consistent with the hypothesis that frequent, close physical contact leads to mite transmission. This hypothesis was supported further by an AMOVA based on the mites isolated from hosts within the three families (Fig. S3), which showed that 20.2% of the molecular variance segregated among families, a result that was unlikely to have occurred by chance ($\Phi_{CT} = 0.202$, $P = 0.03$) (Table S1).

On the other hand, 23.3% of the molecular variance segregated among hosts within each family, a result that also was unlikely to have occurred by chance (AMOVA; $\Phi_{SC} = 0.292$, $P < 0.00001$) (Table S1). Apparently, close physical contact among hosts does not necessarily result in uniform mite populations. This result could be caused by genetic differences among hosts selecting for different mite genotypes; alternatively, it could be caused by differential colonization of each host by mites from elsewhere in the environment, especially given that the offspring in this study were adults and thus likely were exposed to environments increasingly distinct from those of the parents. Studies tracking *Demodex* populations over years on people who move to new countries or who establish new intimate relationships with partners hosting other *D. folliculorum* haplotypes will further clarify the conditions under which mites are transferred between hosts.

The transfer of *Demodex* mites between individuals appears to happen less frequently than the transfer of lice (*Pediculus humanus*), another human-associated arthropod species, as would be expected considering the more external habitat of lice in comparison with these pore-dwelling mites. *D. folliculorum* exhibited greater haplotype diversity than *P. humanus* (30): We recovered 119 haplotypes from only 232 sequences (Table 1), and only 14 of these haplotypes were shared. With relatively few exceptions, most individuals sampled here hosted mites with unique haplotypes, and the sharing of between hosts occurred much more often within family units.

To understand whether *D. folliculorum* divergence corresponds to specific events during human evolution, such as ancient migrations, investigation into the divergence dates of different lineages within *D. folliculorum* is needed. Information required to constrain such an analysis, such as fossil data or rates of molecular evolution for *Demodex*, are unknown. In lieu of such information, we used a strict clocklike analysis based on a rate of mitochondrial evolution commonly applied to arthropods (31) to estimate the divergence times for the major mitochondrial clades (Table S2). These results indicated that the major mitochondrial clades diverged in the distant past. For example, we estimated that the time back to the most recent common ancestor of mitochondrial clades A, B, and C is more than 3 Mya, with a 95% highest posterior density (HPD) interval of 2.4–3.8 Mya. This date roughly corresponds with the origin of the genus *Homo* and is consistent with

the emerging picture of *D. folliculorum* as a species that has a large effective population size and that has been associated with the human lineage for an extremely long period. However, a caveat to these dating results is that *D. folliculorum* evolution likely does not conform to a standard rate of arthropod evolution. Other parasitic arthropods have been found to exhibit elevated rates of molecular evolution (32). If such were the case for *D. folliculorum*, then the actual divergence times between lineages could be much more recent than found here. However, a rate 10 times as fast would still place *D. folliculorum* lineage divergences more than 200,000 y ago, before the estimated origin of modern *H. sapiens*. As more molecular markers are sequenced from *D. folliculorum*, further testing of population divergence times with respect to major events in ancient human history will be compelling. Until then, plausible scenarios indicate that *D. folliculorum* has been with us since our earliest days.

Overall, our results are reconcilable with a model of mite phylogeography in which *D. folliculorum* originated with humans in Africa and then diverged among populations of their descendants as they migrated across the globe. In some cases, the association between host regional ancestry and their mite lineages appeared to persist over generations of living in another region of the world. Additional sampling of humans from a variety of biogeographic ancestries will be necessary to unravel the story of *D. folliculorum* evolution. In particular, more sampling among people from multiple regions in Africa is likely to contribute greatly to our understanding of the history and diversity of *D. folliculorum*; because nearly all human genetic diversity is found in Africa (33), much of the diversity of human-associated *Demodex* may be found in Africa as well.

The patterns of divergence we found among *Demodex* mites associated with human hosts contribute to a growing literature on the phylogeography of human-associated species (1, 7, 10). Humans have spread around the world, accompanied by microbes and metazoans in and on our bodies as well as the many species associated with human dwellings and agriculture. These organisms are indicative of the human story because their relatively rapid generation times compared with their hosts lead to faster accumulation of mutations and potentially a more detailed molecular recording of human movement. Considering the ancient divergences within *D. folliculorum*, and the nearly universal presence of *Demodex* on adult humans, these mites provide an excellent system for studying past and present relationships among human populations.

Materials and Methods

The work presented here represents two sets of data collected independently using complementary methods and then combined to provide a more robust basis for analysis than would be possible by relying on either dataset alone. Methods for the initial biological sample collection and sequence processing were distinct for each dataset before analyses.

Ethics Statement. All participants were sampled by project authors or associated project staff. Potential participants were informed about the goals of the project and the sampling protocols. Those who agreed to participate signed informed consent forms and answered brief questionnaires. Sampling procedures, questionnaires, and participant informed consents were approved by either the North Carolina State University's Human Research Committee (Approval no. 2966) or the Bowdoin College Research Oversight Committee (Approval no. 2007-34).

Sampling and DNA Extractions. Sampling of *D. folliculorum* was performed by one of two methods. Intact mites were isolated from 31 participants who provided information about their geographic region of birth, regional ancestry, and, in some cases, specified their country of birth (Table S3). Mites were collected by drawing the curved end of a bobby pin across the forehead of each participant. We examined the resulting exudates for *Demodex* mites, finding 179 intact mites from these participants. The mites were washed several times in fresh mineral oil; then the mineral oil was removed by washing 10 times with 100% ethanol before DNA extractions. The ethanol was evaporated by heating for 2 min at 95 °C; then the dried mites were suspended in 10 μL lysis buffer (1 μL of 10X PCR buffer, 0.8 units Proteinase K in 1 μL H_2O, 8 μL 1% Triton X) and were incubated 60 min at 65 °C followed by 10 min at 95 °C, frozen at −20 °C for at least 1 h, and stored at −20 °C until used for PCR.

Details about where they had lived and their ancestry were collected from 39 participants (Table S3); this information included their ancestral geographic origins, country of birth, current country of residence, and the countries where their parents were born. Rather than isolating individual mites from these participants, we scraped their cheeks and nasolabial folds with metal laboratory spatulas, as described previously (14). The entire quantity of exuded sebum and associated material (e.g., hair and skin cells) was used for DNA extractions, regardless of the presence of intact mites. For these DNA extractions, we used either a Qiagen DNeasy Blood & Tissue kit or the Omega Bio-Tek E.Z.N.A Tissue DNA kit. The final DNA elutions were performed with 100 μL of elution buffer. The eluted DNA samples were stored at −20 °C until later use for PCR. Altogether, 58 DNA sequences were isolated from these 39 participants using these methods.

Mothers, fathers, and adult offspring from three family units of European ancestry were sampled (offspring/mother/father: hosts 895/872/206, 955/879/677, and 841/505/246). Two of the adult offspring (hosts 677 and 246) were 22 y old when sampled; the third (host 206) was 44–46 y old (this host was sampled on three successive years, 2007–2009).

Amplification and Sequencing. We designed PCR primers for this study to amplify a 930-bp fragment of the mitochondrial genome spanning most of COIII, all of tRNA-Gly, and the beginning of ND3 based on the sequence determined as part of the complete mitochondrial genome of *D. folliculorum* (12): CoIII-PF1: 5′-CATGACCCATCATCTCATCCATC-3′ and ND3-PR2: 5′-CGAAGGGTGAATTTAAGCTGGAAG-3′. We carried out PCRs in 15-μL or 50-μL volumes containing 5–20 ng of template DNA and 0.1 μM of each primer in deionized water. A touchdown PCR cycling program was used, with three cycles each with annealing temperatures of 52 °C, 51 °C, and 50 °C followed by 29 cycles with an annealing temperature of 49 °C. The PCR products were purified and sequenced. In many cases in which total sebum, rather than an isolated mite, was used for DNA extractions, the resulting PCR produced overlapping sequencing, indicating the presence of multiple DNA sequences from more than one mite. In these cases the PCR products were cloned using a TOPO TA cloning system (Invitrogen), and several clones were sequenced to isolate distinct sequences from individual mites.

All *Demodex* sequences were edited, aligned, and trimmed to a common length using Clustal W (34). The alignments were confirmed visually. No indels or frameshift mutations were detected.

Population-Level Analyses. Haplotype (h) and nucleotide (π) diversities were obtained with Arlequin v. 3.5.1. The nucleotide substitution model used to calculate genetic distance was Kimura 2-Parameter + $\Gamma = 0.024$, the best-fit model indicated by the corrected Akaike information criterion method in jModelTest 2 v. 2.1.5 (35, 36).

To investigate whether the genetic variation in *Demodex* mtDNA was structured according to the geographic origin of human host ancestries, we grouped mite sequences ($n = 232$) by the self-reported regional ancestry of their human hosts: Europe, Africa, Asia, or Latin America (Table S2). We applied an AMOVA based on Φ_{ST}, an analog of Wright's F_{ST} that incorporates a model of sequence evolution (37). We applied nonparametric procedures to generate a null distribution and to test the significance of the variance components for each hierarchical comparison (10,000 iterations).

To test for the stability of the host–mite association through time, we analyzed sequence data ($n = 36$) from mites sampled from a single individual (host 206) over the course of three consecutive years (2007–2009). We estimated Φ_{ST} based on the Tamura and Nei (38) model of evolution, which was the best model of evolution indicated by jModelTest 2 v. 2.1.5 among those implemented in Arlequin. We applied an AMOVA analysis to test the significance of variance components for comparisons among years.

To test whether mites tend to be transmitted from parents to offspring and between spouses, we analyzed mtDNA sequence data obtained from mites sampled on three family units (mother/father/offspring). We estimated Φ_{ST} based on the Tamura and Nei (38) model and applied AMOVA analyses to test the significance of variance components for comparisons among host families and among hosts within families.

To gain insight into the evolutionary history of *D. folliculorum*, we conducted Fu's F_s test (39) for departure from the mutation-drift equilibrium model with Arlequin. Large and negative values of Fu's F_s are expected in populations that have experienced recent expansions or selection. We also investigated the historical demography of *D. folliculorum* by calculating Harpending's raggedness index r (40); nonsignificant raggedness scores suggest recent (and rapid) population expansion.

Phylogenetic Analyses. Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference. ML analyses were conducted using the GTR-Γ model implemented in RAxML v. 7.2.6 (41), which estimates and optimizes each partition for individual α-shape parameters, general time-reversible (GTR) rates, and empirical base frequencies. ML bootstrap analyses (10,000 replicates) used the same model and search options as above. A posteriori bootstrapping analysis conducted with RAxML's autoMRE tool indicated that trees converged after 1,000 replicates. All analyses were performed on a 280-core Apple Xserve Xeon cluster using the iNquiry bioinformatics cluster tool (version: 2.0, build: 755). Bayesian analysis was done with MrBayes (42). Analysis was partitioned by codon position (three partitions: pos 1, pos 2, and pos 3) and run with a GTR +I+Γ model. Two independent runs were performed for 10 million generations, each with four chains (three heated and one cold), uninformative priors, and trees sampled at intervals of 1,000 generations. Stationarity was determined by examining the SD of split frequencies between the two runs for convergence. Burn-in fraction was set at 0.25, and remaining trees were used to construct a 50% majority rule consensus tree. To visualize the conflicting phylogenetic signal in our dataset, we constructed a Neighbornet network in Splitstree (43), with variance set to Ordinary Least Squares.

Clade Divergence Estimates. We used BEAST 1.8.2 (44) to estimate the times of divergence among the four major *D. folliculorum* mitochondrial clades. We applied a strict clock sampling from a uniform distribution and a Yule process speciation model, a GTR substitution model estimating base frequencies, and a Gamma site heterogeneity model. The molecular evolutionary rate of *Demodex* is unknown. We chose the rate of 1.1 ± 0.3% per million years based on a rate of mitochondrial evolution commonly applied to arthropods (31). We performed four independent runs of 250 million generations sampling every 250,000 generations. We removed 10% of the initial samples as our burn-in. The log and tree files from each run were combined using Logcombiner v.1.8.2 for a total of one billion generations. We used Tracer 1.6 (44) to evaluate the BEAST log files to confirm convergence and assess the effective sample size values.

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