Evidence for a bacterial mechanism for group-specific social odors among hyenas

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Symbiotic microbes can benefit their animal hosts by enhancing the diversity of communication signals available to them. The fermentation hypothesis for chemical recognition posits that 1) fermentative bacteria in specialized mammalian scent glands generate odorants that mammals co-opt to communicate with one another, and 2) that variation in scent gland odors is due to underlying variation in the structure of bacterial communities within scent glands. For example, group-specific social odors are suggested to be due to members of the same social group harboring more similar bacterial communities in their scent glands than do members of different social groups. We used 16S rRNA gene surveys to show that 1) the scent secretions of spotted hyenas are densely populated by fermentative bacteria whose closest relatives are well-documented odor producers, and that 2) these bacterial communities are more similar among hyenas from the same social group than among those from different groups.

The evolutionary history of animals spans 600 million years, and over its course no animal has evolved independently of symbiotic microbes1,2. Instead, each animal has maintained intimate associations with microbial symbionts that have likely had profound effects on the animal’s biology1–3. Associations between animals and their symbiotic microbes can certainly be antagonistic, but many appear to be mutualistic. For instance, animal-associated bacteria effectively prime host immune systems, competitively exclude host pathogens, afford hosts access to otherwise inaccessible vitamins and nutrients, and facilitate the development and functioning of host tissues4,5. It is also becomingly increasingly clear that symbiotic bacteria can beneficially affect animal behavior, and that their contributions may be particularly prominent in the realm of chemical communication6. Communication via chemical means appears to be the oldest and most widespread mode of signaling among animals7,8. The components of animal chemical signals can be synthesized by signalers themselves, obtained directly from signalers’ environments, or acquired from the metabolic products of symbiotic microbes9. Many mammals communicate chemically by scent marking with secretions from specialized integumental scent glands2–10. These scent glands are warm, moist, organic-rich and largely anaerobic, and thus appear highly conducive to the proliferation of fermentative symbiotic bacteria10.

The fermentation hypothesis for chemical recognition, introduced over 30 years ago, posits that, as bacteria ferment the protein and lipid-rich substrates in scent glands, they produce odorous metabolites that are co-opted by their mammalian hosts as components of chemical signals6,10. The hypothesis further posits that variation in many mammalian chemical signals, both among and within species, is due, at least in part, to underlying variation in the composition or structure of odor-producing bacterial communities within scent glands. For example, individual-specific scents are postulated to be a product of individuals harboring unique bacterial communities in their scent glands, whereas group-specific scents—which are not mutually exclusive of individual-specific scents—are suggested to be due to members of the same social group harboring more similar odor-producing bacterial communities in their scent glands than do members of different social groups6,10. Group-specific bacterial communities could arise through cross-infection among group members, as a consequence of members occupying the same space, coming into frequent bodily contact with one another, and/or consistently scent marking the same sites (e.g. communal scent posts). This mechanism was recently proposed as an explanation for the existence of observed group-specific social odors in the big brown bat, Eptesicus fuscus, Bechstein’s bat, Myotis bechsteinii, European badger, Meles meles, and the spotted hyena, Crocuta crocuta11–15. From a functional standpoint, group-specific odors could facilitate rapid discrimination of group members from others, thereby enabling appropriate conciliatory or agonistic responses to encountered conspecifics6,16.
If the fermentation hypothesis for group-specific social odors among scent-marking mammals is correct, then minimally 1) mammalian scent glands should contain substantive populations of odor-producing bacteria, and 2) the composition and/or structure of bacterial communities inhabiting the scent glands of members of the same social group should be more similar than those associated with members of different social groups. Historically, technical limitations associated with cultivation-based surveys of symbiotic bacteria have impeded the ability to effectively test predictions of the fermentation hypothesis for chemical recognition and, as a consequence, evaluations of the hypothesis have typically yielded either ambiguous results or concluded that the bacterial diversity in integumental scent glands is insufficient to underlie the observed diversity in chemical signals. However, contemporary culture-independent, molecular survey tools can more effectively elucidate the diversity and metabolic potential of symbiotic bacterial communities directly from nucleotide sequences. The objective of the current study was to use sequence-based community survey tools to evaluate first predictions of the hypothesis that symbiotic bacterial communities underlie the existence of group-specific social odors among wild spotted hyenas.

Spotted hyenas are large carnivores found throughout sub-Saharan Africa. They live in complex social groups, called clans, which typically consist of 40-80 individuals. Clans contain multiple breeding males and multiple overlapping generations of females. The adult members of each clan cooperatively maintain and defend their group’s territory against neighboring hyena clans, and they also direct hostility toward same-sex foreign hyenas intruding into their group’s territory. Despite being cohesive units, hyena clans are fission-fusion societies, in which members seldom all occupy the same place at the same time. Instead, subgroups of clan members form and dissolve such that these subgroups change in size and composition several times per day. To mediate the complex social relationships both within and among clans, spotted hyenas employ a rich suite of tactile, visual, vocal and chemical signaling behaviors. A particularly common and conspicuous chemical signaling behavior among hyenas is ‘pasting,’ a form of scent marking wherein a hyena typically straddles a grass stalk, extrudes its anal scent pouch, and drags the exposed pouch across the top of the stalk, leaving behind a thin layer of secretion, called ‘paste.’ Paste is composed of lipid-rich sebum and presumably desquamated epithelial cells, and it is produced by a pair of lobulated sebaceous glands that secrete their products directly into the anal scent pouch.

The major volatile constituents of paste are fatty acids, esters, hydrocarbons, alcohols and aldehydes. Collectively, they give paste a pungent, sour mulch odor that persists, detectable by the human nose, for more than a month after paste is deposited on grass stalks. Previous investigations have shown that the odor of spotted hyena paste varies based on the individual identity, sex and group membership of the scent donor. Hyenas’ group-specific odors, in particular, are due to underlying variation in the structure of short-chain fatty acid and ester profiles of paste. These odorants are well-documented products of bacterial fermentation. Therefore, it has been suggested that group-specific social odors among spotted hyenas are a product of hyenas harboring group-specific bacterial communities in their scent pouches. Here we initiate evaluation of this hypothesis.

This study provides the first in-depth, next-generation sequencing survey of the bacterial communities inhabiting the specialized scent glands of any mammal. It illustrates that individual glands can support far greater bacterial diversity than was previously reported, and that, among hyenas, this diversity comprises bacteria whose closest relatives are well-documented odor producers. Furthermore, by evaluating the diversity of bacterial communities in the scent glands of hyenas belonging to multiple clans in an African Reserve (Figure 1), this study provides the first cultivation-independent test of the prediction that the structure of bacterial communities in the scent glands of group-mates should be more similar than those among members of different social groups. The data support these predictions and, therefore, also the fermentation hypothesis for group-specific chemical recognition among spotted hyenas.

**Results**

**Characterization of scent pouch bacterial communities.** Scanning electron micrographs (SEMs) revealed that spotted hyena paste...
supports substantive populations of coccus and rod-shaped bacteria, and that some of these bacteria appeared to be in the process of dividing when sampling occurred—as indicated, for instance, by the presence of fission rings among rods (Figure 2). Subsequent 16S rRNA gene surveys of the bacterial communities in paste further revealed that these bacteria are primarily members of the phyla Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria (Table 1). The sampled pouch bacteria represented 78 previously characterized genera but, importantly, this is a minimum estimate of genus-level bacterial richness because more than half of the sampled sequences could not be confidently (>80% confidence threshold) assigned to a pre-established bacterial genus, suggesting the presence of many bacterial types new to science. We found 16 prominent operational taxonomic units (OTUs)—each constituting, on average, at least 0.5% of sampled sequences—in the scent pouches of female spotted hyenas. Collectively, these 16 OTUs accounted for more than 90% of the sampled sequences, and seven of these OTUs were found in the pouches of all 16 hyenas. When representative 16S rRNA gene sequences of these prominent OTUs were integrated into a phylogenetic tree alongside those of neighboring bacterial type strains, it became evident that the scent pouches of female hyenas generally contained the same bacterial OTUs, regardless of which clan the hyena belonged to. There was, however, an evident effect of clan membership on the structure of these symbiotic communities, with the relative abundances of OTUs being more similar among clan-mates than among hyenas from different clans (Table 2; Figure 4). The degree of variation in the structure of scent pouch bacterial communities within clans did not significantly vary among clans, suggesting that the clans’ symbiotic communities varied in their multivariate locations, not their dispersions (permutation analysis of multivariate dispersions, PERMDISP2; Bray-Curtis; distance to spatial medians; 9999 permutations of the least-absolute-deviation (LAD) residuals; P = 0.106). Specifically, the bacterial communities inhabiting the pouches of Southern Comfort, Mara River and Fig Tree hyenas were distinguishable. The structure of the scent pouch communities of Emarti Hill hyenas were different from those of Southern Comfort hyenas, but were not consistently different from those of Mara River or Fig Tree hyenas. Similarity Percentage (SIMPER) analyses indicated that OTUs 1, 4, 6, 7, 9, 55 and 63 were largely responsible for the observed differences in scent pouch bacterial communities among the hyena clans (Table 3). Each of these OTUs is a prominent member of hyena scent pouch bacterial communities (Table 2). In other words, the scent pouches of female hyenas generally contained the same bacterial OTUs, regardless of which clan the hyena belonged to. There was, however, an evident effect of clan membership on the structure of these symbiotic communities, with the relative abundances of OTUs being more similar among clan-mates than among hyenas from different clans (Table 2; Figure 4). The degree of variation in the structure of scent pouch bacterial communities within clans did not significantly vary among clans, suggesting that the clans’ symbiotic communities varied in their multivariate locations, not their dispersions (permutation analysis of multivariate dispersions, PERMDISP2; Bray-Curtis; distance to spatial medians; 9999 permutations of the least-absolute-deviation (LAD) residuals; P = 0.106). Specifically, the bacterial communities inhabiting the pouches of Southern Comfort, Mara River and Fig Tree hyenas were distinguishable. The structure of the scent pouch communities of Emarti Hill hyenas were different from those of Southern Comfort hyenas, but were not consistently different from those of Mara River or Fig Tree hyenas. Similarity Percentage (SIMPER) analyses indicated that OTUs 1, 4, 6, 7, 9, 55 and 63 were largely responsible for the observed differences in scent pouch bacterial communities among the hyena clans (Table 3). Each of these OTUs is a prominent member of hyena scent pouch bacterial communities (Figure 3). Aside from OTU 1, which is a Propionibacterium, the others are generally unclassified Firmicutes.

**Discussion**

The fermentation hypothesis for chemical recognition predicts that specialized mammalian scent glands 1) harbor odor-producing...
bacteria, and that 2) the structure of bacterial communities in these glands varies with the host trait of interest—here, clan membership. The data from scanning electron microscopy (SEM) were consistent with the prior suggestion that, similar to the scent pock secretions of many other mammals, hyena paste is a mixture of lipid-rich sebum and desquamated epithelial cells. The micrographs also revealed that paste supports substantive communities of coccus and rod-shaped bacteria and, furthermore, provided evidence, such as fission rings, that some of these bacteria were in the process of dividing, and were therefore necessarily metabolically active when paste sample collection occurred. The 16S rRNA gene surveys of paste revealed that more than half of the analyzed sequences could not be confidently assigned to a previously characterized genus. The hyena scent pock microflora therefore contains many novel bacterial types. Still, the 16S sequences that could be assigned to a genus represented 78 genera from nine different bacterial phyla. Collectively, the 15 prior surveys of specialized mammalian scent glands—covering 14 species from five mammalian orders—recovered bacteria from just 63 genera within five bacterial phyla. Therefore, as expected, the next-generation sequencing approach used in this study afforded a far more comprehensive view of the bacterial communities inhabiting the specialized scent glands of a mammal than was previously available and, consequently, overturned the general conclusion from earlier studies that the bacterial diversity in integumental scent glands appears insufficient to underlie the observed diversity of chemical signals within scent marking mammalian species.

In this study, the prominent OTUs—some assigned to a bacterial genus, some not—were incorporated into a phylogenetic tree to gain additional taxonomic information about the hyena scent pock microflora. The phylogenetic analysis showed that the scent pock microflora are largely obligate or facultative anaerobes whose closest characterized relatives are well-documented odor producers. Specifically, the pouch bacteria belong, or are closely related, to genera that produce a diverse array of short-chain fatty acids (see Supplementary Table S1 online). Consistent with the fermentation hypothesis for chemical recognition, there is also documented variation in SCFA production at the species/strain level within these genera. Therefore, variation in the structure of symbiotic communities composed of these bacteria could very feasibly result in variation in host odor profiles.

SCFAs are prominent odors in spotted hyena paste. Furthermore, they largely constitute group-specific paste odors among hyena clans. Group-specific paste odors, similar to the group-specific social odors of other mammals, and even soil insects, are due to variation in the structure of odor profiles among social groups. In this study, there was a significant effect of clan membership on the structure of bacterial communities among social groups. In this study, there was a significant effect of clan membership on the structure of bacterial communities among social groups. In this study, there was a significant effect of clan membership on the structure of bacterial communities among social groups. In this study, there was a significant effect of clan membership on the structure of bacterial communities among social groups. In this study, there was a significant effect of clan membership on the structure of bacterial communities among social groups.

Table 1: Phylum and genus-level assignments of operational taxonomic units (OTUs) in the scent pouches of female hyenas

| Phylum      | Number of OTUs | Percentage of Sequences (± SD) | Genera                                      |
|-------------|----------------|-------------------------------|---------------------------------------------|
| Firmicutes  | 236 (55)       | 73.3 ± 13.9 (22.9 ± 14.6)     | Anaerococcus, Anaerovorax, Bacillus, Blautia, Clostridium, Coprobacillus, Dialister, Eubacterium, Packalonia, Finegoldia, Fusibacter, Helcococcus, Hedeomycetes, Ignovirgynum, Macrooccus, Peptococcus, Peptinophilus, Peptostreptococcus, Sedimentibacter, Sporosarcetigem, Staphylococcus, Streptococcus, Tissierella |
| Actinobacteria | 53 (34)       | 17.1 ± 14.0 (17.0 ± 14.0)     | Actinomyces, Arthrobacter, Blastosaccus, Brachybacterium, Cellulomonas, Corynebacterium, Curtobacterium, Dietzia, Friedmanniella, Janibacter, Kinesorbo, Marmorocila, Micromonaspora, Olsenella, Phycococcus, Propionibacterium, Rubrobacter, Virgisorpinium |
| Bacteroidetes | 27 (14)        | 3.1 ± 3.2 (3.1 ± 3.1)         | Bacteroides, Flavisolbacter, Pelodictobacter, Parabacteroides, Paraprevotella, Pedobacter, Petromonas, Porphyromonas, Prevotella |
| Proteobacteria | 27 (23)        | 1.1 ± 3.1 (1.0 ± 3.1)         | Acinetobacter, Aeromonas, Anaeromyxobacter, Arcobacter, Bradyrhizobium, Comamonas, Enhydrobacter, Herbaspirillum, Massilia, Neisseria, Novosphingobium, Pantoee, Phenyllobacterium, Pseudomonas, Sphingomonas, Stenotrophomonas, Sutterella |
| Chloroflexi  | 13 (3)         | 0.1 ± 0.1 (< 0.1 ± 0.0)       | Gemmatimonas |
| Fusobacteria | 4 (3)          | 0.1 ± 0.3 (0.1 ± 0.3)         | Gemmatimonas, Fusobacterium |
| Acidobacteria | 6 (6)          | < 0.1 ± 0.0 (< 0.1 ± 0.0)     | Gp4, Gp6, Gp7, Gp17 |
| Tenericutes  | 1 (1)          | < 0.1 ± 0.0 (< 0.1 ± 0.0)     | Actinobacter, Anaerovorax, Actinobacteria, Anaeromyxobacter, Arcobacter, Bradyrhizobium, Comamonas, Enhydrobacter, Herbaspirillum, Massilia, Neisseria, Novosphingobium, Pantoee, Phenyllobacterium, Pseudomonas, Sphingomonas, Stenotrophomonas, Sutterella |
| Gemmatimonadetes | 1 (1)       | < 0.1 ± 0.0 (< 0.1 ± 0.0)     | Gemmatimonas |
| Classified Totals | 368 (140) | 94.8 ± 4.1 (44.1 ± 24.5) | 78 Characterized Genera |

Taxonomic assignments of OTUs were determined using the Ribosomal Database Project’s Classifier tool with an 80% confidence threshold. Most of the sequences that remained unclassified at the phylum level belonged to OTU 21 (see Figure 3), which fell just short of being placed in the phylum Firmicutes with a confidence of 79%.
is low, it is higher within than among clans\textsuperscript{39,40}. Given that the bacterial communities in the urine marks of laboratory mice vary with the major histocompatibility complex haplotype and broad background genotype of the host\textsuperscript{35,36}, similar effects of these factors on the scent pouch bacterial communities of hyenas ought to be considered as well. Importantly, however, the underlying mechanisms for the formation of bacterial communities in the scent glands of mammals, although deserving of further investigation, are ancillary to the evaluation of the fermentation hypothesis for group-specific chemical recognition presented here.

This study employed scanning electron microscopy and next-generation sequencing technology to test necessary first predictions of the fermentation hypothesis for group-specific chemical recognition in the spotted hyena. It demonstrated that the scent pouches of spotted hyenas harbor diverse communities of anaerobic, fermentative bacteria whose close relatives are known odor-producers. Furthermore, the odors they produce are the same odors shown to constitute group-specific social odors among hyena clans. This study also revealed the existence of group-specific bacterial communities in the scent pouches of hyenas, illustrating that their diversity is sufficient to underlie group-specific social odors in this mammal. It does not, however, definitively demonstrate that they do so. Therefore, we are currently testing further predictions of the fermentation hypothesis for chemical recognition in the spotted hyena. Specifically, we
are testing the predictions that the odor and bacterial profiles of individual paste samples co-vari, and that specific members of the scent pouch microbiota, isolated and grown in pure culture, generate SCFAs and other odorants found in paste.

Methods

Collection of scent pouch secretions (called ‘paste’). From 1999 – 2000, paste samples were collected directly from the anal scent pouches of 16 lactating female hyenas in the Masai Mara National Reserve, Kenya. Each had been anesthetized using Telazol (6.5 mg/kg) delivered from a CO₂-powered darting rifle. The paste samples were placed in sterile cryogenic vials, stored in liquid nitrogen, and transported to Michigan State University, where they remained frozen at −80 °C until being used in this study. The sampled females represented four distinct hyena clans within the north-central region of the Reserve: Southern Comfort, Fig Tree, Mara River and Emarti Hill (Figure 1).

Scanning electron microscopy. Paste samples were fixed for 1 hr in 4% glacial acetic acid buffered with 0.1 M sodium phosphate at pH 7.4. They were then rinsed and postfixed in 1% osmium tetroxide for 1 hr, before being rinsed again and dehydrated in an ethanol series (25%, 50%, 75%, 95% vol/vol) for 15 mins at each step, followed by 30 cycles of 95 °C for 20 s (denaturation), 50 °C for 30 s (annealing) and 72 °C for 5 minutes (extending). Each sample was PCR-amplified in duplicate and each amplification reaction included the incorporation of a unique molecular barcode to enable multiplex sequencing of samples41. Following amplification, PCR products were purified using solid-phase reversible immobilization (SPRI bead clean-up; Agencourt AMPure XP), quantified using an Inivitrogen high sensitivity Quant-iT dsDNA assay kit, and pooled at equimolar concentrations. Nucleotide sequencing was performed on a 454 GS FLX Titanum instrument (Roche Diagnostics) at the Baylor College of Medicine’s Human Genome Sequencing Center.

Sequence processing and operational taxonomic unit (OTU) formation. During initial processing, sequences were culled if they 1) contained any ambiguous base calls, 2) did not have perfect barcode and forward primer matches, 3) did not span the entire 900 – 500 base position region of the Escherichia coli (R16995 16S rRNA gene, or 4) if they did not have a minimum average exponential quality score of 25 or greater over this region. The remaining sequences were truncated at the E. coli positions 900 and 500, which correspond to taxonomically-conserved regions of the 16S rRNA gene46. Sequences were then aligned using the Infernal Aligner tool provided by the Ribosomal Database Project (RDP) and were binned into operational taxonomic units (OTUs)—based on their percent nucleotide sequence similarity—using RDP’s complete-linkage clustering tool47 (v 10.23). In this study, we binned sequences using 90, 97 and 98% sequence similarity cutoffs. Results consequent of a 97% cutoff are presented here, and those from 90 and 98% cutoffs—which tell a very similar story—are included as supplementary materials online (Table S2, Figure S3). For perspective, we note that bacterial 16S rRNA sequences sharing at least 97% of their homologous bases are often referred to as coming from conspecifics, but we caution that the strength and functional relevance of this correlation remains uncertain48.

To minimize the potential influences of PCR bias and sequencing errors on community analyses49, 50, OTUs were culled if they occurred in only a single sequencing reaction, or if their representative sequences were flagged as chimeric by the Chimera Slayer tool in Mothur49 (v 1.17.0, Silva Gold database). A single exception was made for OTU 21 (see Figure 3), which was recovered in high abundance in all sequencing reactions and appeared legitimate when it was manually compared to unflagged OTUs in this study and to 16S rRNA reference sequences using using Arb software50, 51 (v 5.1; Silva rRNA database, v 104). Experimentally removing OTU 21 from our data set did not affect analyses. Lastly, OTUs were culled if the RDP Naïve Bayesian rRNA Classifier tool42 (v 2.2, 80% confidence threshold) deemed them chloroplast DNA sequences. In total, 3.5% (3958/111896) of sequences were culled. When the OTU-calling process was complete, we verified that each sample was most similar to its technical replicate, and then combined the data from each female’s replicate samples, except for hyena EH489, for whom only a single sequencing reaction was successful. Ultimately, 107,938 sequences were binned into 403 OTUs, with each female contributing 6746 ± 1547 sequences representing 19 ± 43 OTUs (means ± SD throughout, unless otherwise noted). The Good’s coverage estimations for the samples were 99.3% ± 0.2, and the rarefaction curves for all samples had plateaued (see Supplementary Fig. S4 online). Together, these data indicated that sample coverage was consistently very high for communities sampled in this study.

Characterizing the taxonomic identities of scent pouch bacteria. Representative sequences of the 403 OTUs were obtained using the Dereplicate tool in the RDP (GenBank: JK051873 – JK052266; see Supplementary Information S5, S6 online). The RDP Naïve Bayesian rRNA Classifier tool was then used to assign phylum and genus-level taxonomic identities to the representative sequences51 (v 2.2, 80% confidence threshold). Even though the RDP Classifier performs particularly well when classifying sequences spanning the V3–V5 region of the 16S rRNA gene46, many of the OTUs in this study could not be confidently assigned to previously characterized genera (see Results). Therefore, to obtain approximate genus-level taxonomic information for the prominent OTUs in female scent pouches, representative sequences were incorporated into a phylogenetic tree alongside sequences from a randomized Accelerated Maximum Likelihood (RAxML) rapid bootstrap algorithm52 (v 7.0.3, filters = positions 10351 – 27583, pos_var_Bacteria_102). The 16 OTUs represented in this tree constitute more than 90% of the sequences analyzed in this study.

16S rRNA-encoding gene amplification and sequencing. DNA was extracted from each paste sample (~ 0.1 g) using a MO BIO UltraClean fecal DNA kit, and each extraction was subsequently diluted tenfold with nuclease-free water. Bacterial 16S rRNA-encoding genes in diluted extractions were amplified and purified by following the provisional protocol of the National Institute of Health’s Human Microbiome Project (http://www.hmpdacc.org/tools_protocols/tools_protocols.php). Specifically, 16S rRNA genes were PCR-amplified using two broadly conserved primers targeting the V3-V5 variable regions of the bacterial 16S rRNA gene (5’ – CCG TCA ATT CMT TTR AGT – 3’; 357F; 5’ – CCT ACG GGA GGC AGG AG – 3’). The PCR program consisted of an initial dissociation step of 95 °C for 2 min, followed by 30 cycles of 95 °C for 20 s (denaturation), 50 °C for 30 s (annealing), and 72 °C for 5 minutes (extending). Each sample was PCR-amplified in duplicate and each amplification reaction included the incorporation of a unique molecular barcode to enable multiplex sequencing of samples41. Following amplification, PCR products were purified using solid-phase reversible immobilization (SPRI bead clean-up; Agencourt AMPure XP), quantified using an Invitrogen high sensitivity Quant-iT dsDNA assay kit, and pooled at equimolar concentrations. Nucleotide sequencing was performed on a 454 GS FLX Titanum instrument (Roche Diagnostics) at the Baylor College of Medicine’s Human Genome Sequencing Center.

Global effect R = 0.17, P = 0.033
SC vs. MR R = 0.31, P = 0.084
SC vs. FT R = 0.19, P = 0.087
SC vs. EH R = 0.41, P = 0.063
MR vs. FT R = 0.05, P = 0.288
MR vs. EH R = 0.01, P = 0.544
FT vs. EH R = 0.00, P = 0.488

Table 2 | Results of analyses of similarity (ANOSIM) evaluating variation in the composition and structure of scent pouch bacterial communities among hyena clans. The abbreviations refer to the sampled clans: SC = Southern Comfort, MR = Mara River, FT = Fig Tree, EH = Emarti Hill.

Figure 4 | Nonmetric multidimensional scaling (NMS) plot illustrating variation in scent pouch bacterial community structure among hyena clans. In NMS plots, the distance of sampled communities to one another reflects their underlying distance in multivariate space—here based on the Bray-Curtis similarity index. The X and Y axes were symmetrical, and the plot’s stress (0.12)—a measure of its goodness-of-fit—indicated that the plot was an informative representation of community-level data45.
Table 3 | Results of SIMPER analyses indicating the contribution of specific operational taxonomic units (OTUs) to observed differences in scent pouch community structure among spotted hyena clans. The abbreviations refer to the sampled clans: SC = Southern Comfort, MR = Mara River, FT = Fig Tree, EH = Emarti Hill

| Clan A vs. B | Overall Average Dissimilarity | Five Most Influential OTUs | Percent Contribution to Difference | Average Percent Abundance in Clan A ± SEM | Average Percent Abundance in Clan B ± SEM |
|-------------|-------------------------------|---------------------------|-----------------------------------|------------------------------------------|------------------------------------------|
| SC vs. MR   | 49.7                          | 7                         | 16.6                              | 6.07 ± 2.41                              | 22.58 ± 2.25                             |
|             |                               | 1                         | 13.8                              | 4.63 ± 1.06                              | 18.63 ± 1.91                             |
|             |                               | 4                         | 12.0                              | 16.53 ± 1.29                             | 4.62 ± 2.55                              |
|             |                               | 6                         | 10.0                              | 11.08 ± 0.42                             | 1.17 ± 0.41                              |
|             |                               | 9                         | 8.9                               | 29.28 ± 2.88                             | 25.96 ± 4.92                             |
| SC vs. FT   | 54.6                          | 4                         | 14.2                              | 16.53 ± 1.29                             | 1.05 ± 0.55                              |
|             |                               | 6                         | 11.2                              | 11.08 ± 0.42                             | 15.50 ± 9.07                             |
|             |                               | 9                         | 7.0                               | 29.28 ± 2.88                             | 18.26 ± 6.30                             |
| SC vs. EH   | 47.7                          | 7                         | 24.6                              | 6.07 ± 2.41                              | 26.14 ± 13.68                            |
|             |                               | 4                         | 11.6                              | 16.53 ± 1.29                             | 5.47 ± 2.46                              |
|             |                               | 1                         | 10.8                              | 4.63 ± 0.66                              | 14.81 ± 3.50                             |
|             |                               | 9                         | 8.7                               | 29.28 ± 2.88                             | 24.10 ± 4.08                             |
| MR vs. FT   | 53.9                          | 63                        | 6.7                               | 1.39 ± 0.72                              | 6.46 ± 5.70                              |
|             |                               | 6                         | 17.3                              | 18.33 ± 1.91                             | 18.67 ± 12.59                            |
|             |                               | 6                         | 14.3                              | 1.17 ± 0.41                              | 15.50 ± 9.07                             |
|             |                               | 7                         | 12.8                              | 22.58 ± 2.25                             | 8.74 ± 2.58                              |
|             |                               | 9                         | 11.0                              | 25.96 ± 4.92                             | 18.26 ± 6.30                             |

Evaluating variation in scent pouch bacterial communities among hyena clans. OTU count data were standardized through conversion into proportions, and the contributions of highly prominent OTUs to quantitative similarity index calculations were tempered by log10 (x + 1) transformations of the data. However, nearly identical results to those presented here were obtained using untransformed proportion data, as well as OTU count data normalized for sample size. Given that the majority of the OTUs in this study could not be confidently assigned to particular known genera (see Results), and that among those which could 16S rRNA gene copy number was largely unknown or variable, it was not possible to adjust OTU data to reflect potential variation in 16S rRNA gene copy number among community members. Therefore, although our OTU data accurately reflect patterns in bacterial community structure among sampled hyenas, they do not necessarily provide definitive information about the absolute or rank abundances of specific bacteria in scent pouch communities.

Variation in the composition (qualitative) and structure (quantitative) of scent pouch bacterial communities was characterized using Sorensen (Dice coefficient) and Bray-Curtis similarity indices, respectively. Community similarities were then visualized through nonmetric multidimensional scaling (NMS) plots, and statistically evaluated via analyses of similarity (ANOSIM). In NMS plots, samples’ vicinity to other samples in portrayable space—typically two dimensions—reflects their underlying similarity in multisivariate space. It is the preferred method for illustrating relationships among ecological communities. ANOSIM is a multivariate, non-parametric permutation test for evaluating differences in community composition or structure between two or more pre-defined treatment groups (e.g. hyena clans). Given the permutational nature of ANOSIM, the application of Bonferroni corrections to planned pairwise comparisons is overly conservative and substantially increases the likelihood of Type II statistical errors, especially when modest sample sizes exist. When differences in bacterial community structure between hyena clans were observed, similarity percentage (SIMPER) analyses were conducted to elucidate the contributions of specific OTUs to those differences. To maximize interpretability, SIMPER analyses were performed using untransformed percent abundance data. All statistical and graphical analyses were completed using the PAST data analysis package (v 2.12).

Animal Use and Care. Our research, described in Animal Research Protocol IACUC 05/11-110-00, was approved most recently on June 15, 2011 by the Institutional Animal Care and Use Committee at Michigan State University, and complies with Kenyan law.

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