Symbiotic bacteria appear to mediate hyena social odors

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Edited by E. Peter Greenberg, University of Washington, Seattle, WA, and approved October 15, 2013 (received for review April 6, 2013)

All animals harbor beneficial microbes. One way these microbes can benefit their animal hosts is by increasing the diversity and efficacy of communication signals available to the hosts. The fermentation hypothesis for mammalian chemical communication posits that bacteria in the scent glands of mammals generate odorous metabolites used by their hosts for communication and that variation in host chemical signals is a product of underlying variation in the bacterial communities inhabiting the scent glands. An effective test of this hypothesis would require accurate surveys of the bacterial communities in mammals' scent glands and complementary data on the odorant profiles of scent secretions—both of which have been historically lacking. Here we use next-generation sequencing to survey deeply the bacterial communities in the scent glands of wild spotted and striped hyenas. We show that these communities are dominated by fermentative bacteria and that the structures of these communities covary with the volatile fatty acid profiles of scent secretions in both hyena species. The bacterial and volatile fatty acid profiles of secretions differ between spotted and striped hyenas, and both profiles vary with sex and reproductive state among spotted hyenas within a single social group. Our results strongly support the fermentation hypothesis for chemical communication, suggesting that symbiotic bacteria underlie species-specific odors in both spotted and striped hyenas and further underlie sex and reproductive state-specific odors among spotted hyenas. We anticipate that the fermentation hypothesis for chemical communication will prove broadly applicable among scent-marking mammals as others use the technical and analytical approaches used here.

animal behavior | animal communication | microbiome

Every animal is populated by communities of microbes that can profoundly affect its biology, often in beneficial ways (1, 2). Indeed, symbiotic microbes are critical contributors to animal nutrition and immune health, and they serve as important catalysts for the effective development and functioning of animal tissues and neural circuitry (2–5). It also is becoming apparent that symbiotic microbes can extend host behavioral phenotypes in beneficial ways, including facilitating their feeding, antipredator, reproductive, and communicative behaviors (6, 7).

An effective communication system is a critical component of an animal's behavioral repertoire, and one way in which symbiotic microbes might contribute to their hosts' behavioral phenotypes is by increasing the diversity and/or efficacy of the signals available to them (6, 8). Most animals communicate to some extent via chemical means, and mammals in particular often rely on odorous secretions from integumental scent glands to signal conspecifics (8–10). These glands occupy myriad locations on mammals' bodies and are typically warm, moist, nutrient-rich, and largely anaerobic. As such, they are conducive to the proliferation of symbiotic, particularly fermentative, bacteria (10). The fermentation hypothesis for mammalian chemical communication posits that as bacteria ferment or otherwise metabolize the nutrient-rich substrates in these glands, they generate odorous metabolites that subsequently are used by their hosts to communicate with conspecifics (6, 10–12). The hypothesis further suggests that variation in chemical signals among mammals with specialized scent glands results largely from an underlying variation in the odor-producing bacterial communities within these glands. If this hypothesis is true, then (*i*) mammalian scent gland secretions should contain fermentative, odor-producing bacteria, (*ii*) the bacterial and odor profiles of secretions should covary, and (*iii*) these profiles should vary with the host characteristics being signaled, such as species identity, group membership, sex, or reproductive state (6).

Effective testing of these predictions of the fermentation hypothesis requires accurate surveys of the bacterial communities in the scent gland secretions of mammals as well as complementary data on the odorant profiles of these secretions. Historically, technical limitations of cultivation-based surveys and, to a lesser extent, molecular fingerprinting surveys, of symbiotic bacteria have impeded our ability to test these predictions effectively because these approaches often underestimate the actual diversity in bacterial communities (6, 13). As a consequence, evaluations of the hypothesis typically have concluded that the bacterial diversity in integumental scent gland secretions is insufficient to underlie the observed diversity of chemical signals (6, 14). In a recent study, we used next-generation sequencing to thoroughly survey the bacterial communities in the scent gland secretions of adult female spotted hyenas, *Crocuta crocuta* (15). That study revealed more types of bacteria than the 15 previous surveys of specialized mammalian scent glands combined and demonstrated that most of these bacteria were members of fermentative, odor-producing clades. It also revealed that the bacterial communities in scent secretions varied among hyena social

Significance

All animals are populated by microbes, and, contrary to popular belief, most microbes appear highly beneficial to their hosts. They are critical in animal nutrition and immune defense, and they can serve as important catalysts for the effective development and functioning of host tissues. It also is becoming increasingly clear that they can contribute to host behavior. It has been hypothesized that one way they do so is by producing the components of chemical signals that animals use to communicate. We tested and confirmed first predictions of this hypothesis in hyenas, demonstrating that the bacterial and odor profiles of hyena scent secretions covaried and that both profiles varied with characteristics of hyenas known to be communicated through their chemical signals.

Author contributions: K.R.T., A.V., K.E.H., and T.M.S. designed research; K.R.T., A.V., J.A.D., K.D.K., E.N.S.-M., and A.P.W. performed research; K.R.T., A.V., and J.A.D. analyzed data; and K.R.T., A.V., J.A.D., A.P.W., K.E.H., and T.M.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. KC705471–KC706325).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1306477110/-/DCSupplemental.

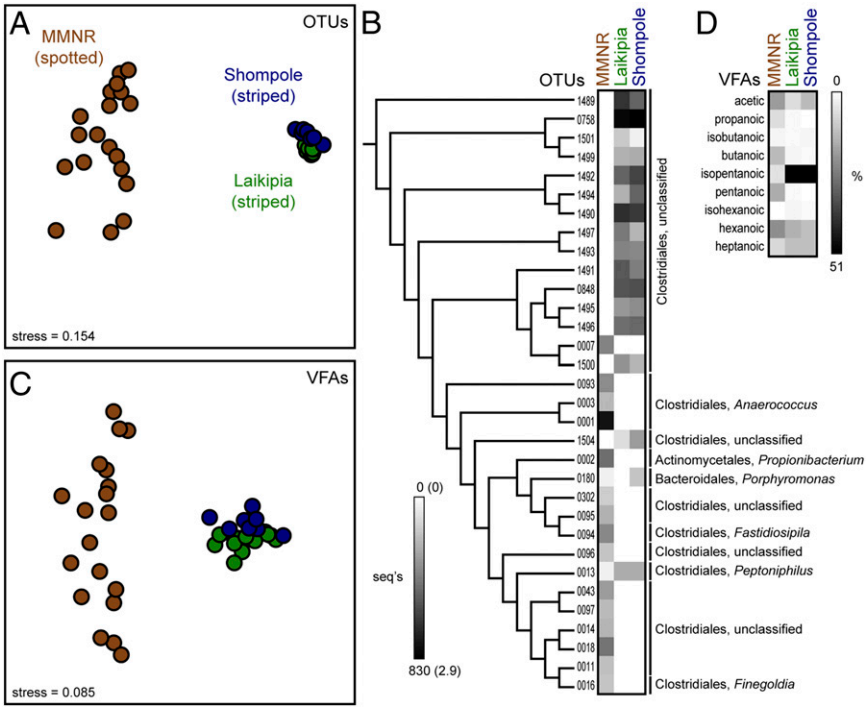

Fig. 1. Differences in the bacterial (OTU) and VFA profiles of the pastes of adult spotted and striped hyenas. (*A*) A nonmetric multidimensional scaling (nMDS) plot showing a difference in structure (Bray–Curtis index) between the paste bacterial communities of adult spotted hyenas from the MMNR and adult striped hyenas from Laikipia and Shompole. (*B*) A Clearcut cladogram (26) of the prominent (i.e., top 15 based on average abundance) OTUs in the pastes of hyenas from the three populations and an accompanying heat map reflecting the mean abundances (out of 1,600 sequences) of these OTUs in pastes. These data were log-transformed before plotting (values in parentheses). Order- and genus-level classifications of OTUs, as determined by the Ribosomal Database Project's Classifier (27), are noted also. (*C*) An nMDS plot showing a difference in structure between the paste VFA profiles of spotted and striped hyenas. (*D*) A heat map of the mean percent abundances of VFAs in the pastes of hyenas from the three populations. Sample sizes were 19, 13, and 9 for MMNR, Laikipia, and Shompole, respectively.

groups, suggesting that their diversity was sufficient to explain social group-specific odors in spotted hyenas (16). Although that study afforded support for the fermentation hypothesis, it did not include complementary data on the odor profiles of hyena scent gland secretions—data needed to evaluate the hypothesis effectively—and the scope of host traits considered was limited. Here we concurrently analyze the bacterial and odor profiles of scent gland secretions collected from wild spotted and striped hyenas, *Hyaena hyaena*, in Kenya (Fig. S1) to determine whether the two profiles covary in each species and to ascertain the extent to which the two profiles vary with hyena species, sex, and, in the spotted hyena, female reproductive state.

The lifestyles of spotted and striped hyenas differ greatly. Spotted hyenas—found throughout sub-Saharan Africa—live in large, hierarchically structured groups, called clans, that typically contain 40–80 individuals (17). Clans include multiple breeding males and multiple overlapping generations of females, and adult members cooperatively maintain and defend their group's territory against neighboring clans (18). To mediate the complex social relationships within and among clans, spotted hyenas use a rich repertoire of tactile, visual, vocal, and chemical signaling behaviors (19, 20). In contrast, striped hyenas—found in North, West, and East Africa—live in small groups containing one or two reproductively mature females and one or more adult males (21). Although the home ranges of group members overlap considerably, striped hyenas usually rest, travel, and forage alone; therefore they seldom interact directly with groupmates (21). Little is known of striped hyena communicative behavior, especially in natural populations, but striped hyenas appear to have a very modest vocal signaling repertoire, with no long-distance vocalizations (22). Therefore, among striped hyenas, chemical signaling likely serves a prominent role in territorial behavior and potentially in reproduction as well.

Despite their very different lifestyles, spotted and striped hyenas both commonly exhibit a conspicuous chemical signaling behavior called "pasting," a form of scent marking in which a hyena deposits an odorous secretion, called "paste," from its subcaudal scent pouch on a grass stalk (20, 22). The major volatile constituents in paste are volatile fatty acids (VFAs), esters, hydrocarbons, alcohols, and aldehydes (23, 24). Previous investigations have shown that the odors of spotted hyena pastes vary with individual identity, group membership, sex, and, potentially, female reproductive state (16, 24, 25). Effects of striped hyena traits on paste odors have not yet been investigated.

This study of mammalian scent marking marries data from in-depth, next-generation bacterial surveys with targeted odor analyses of scent secretions from natural populations. We show that the bacterial communities in hyena pastes are dominated by fermentative bacteria and that the structures of these communities covary with the VFA profiles of pastes. Furthermore, we show that the bacterial and VFA profiles of paste differ between spotted and striped hyenas and that, among spotted hyenas in the same social group, both profiles vary with hyena sex and reproductive state. As such, this study illustrates that the diversity of symbiotic bacterial communities in paste appears sufficient to underlie chemical signaling of host traits in hyenas and affords strong empirical support for the fermentation hypothesis for chemical communication.

Results

The Bacterial and VFA Profiles of Adult Spotted and Striped Hyena Pastes Are Markedly Different. Scanning electron micrographs confirmed that the scent glands of spotted and striped hyenas were inhabited by symbiotic (i.e., resident) microbes (Fig. S2). Subsequent 16S rRNA gene surveys using an operational taxonomic unit (OTU) definition of 97% homologous nucleotide base similarity revealed that the bacterial communities in spotted and striped hyena pastes were markedly different but that each was dominated by fermentative anaerobes.

The structure of bacterial communities in adult spotted hyena pastes differed markedly from those in the pastes of adult striped hyenas (Fig. 1 *A* and *B* and Dataset S1 *A* and *C*) sampled either in Laikipia [analyses of similarity (ANOSIM), $R = 1.0$, $P = 0.0001$] or Shompole ($R = 1.0$, $P = 0.0001$). There was much greater variation in the structure of bacterial communities among the pastes of spotted than striped hyenas [permutational tests of multivariate dispersions (PERMDISP); Masai Mara National Reserve (MMNR) (0.387 ± 0.092) vs. Laikipia (0.131 ± 0.023), $P = 0.0001$; MMNR vs. Shompole (0.144 ± 0.030), $P = 0.0001$; Fig. 1*A*)]. The paste bacterial communities of spotted hyenas also were more OTU-rich (Chao1 index; $t_{\text{unequal var.}} = 2.55$, $P = 0.018$; Table S1). Last, membership in paste bacterial communities

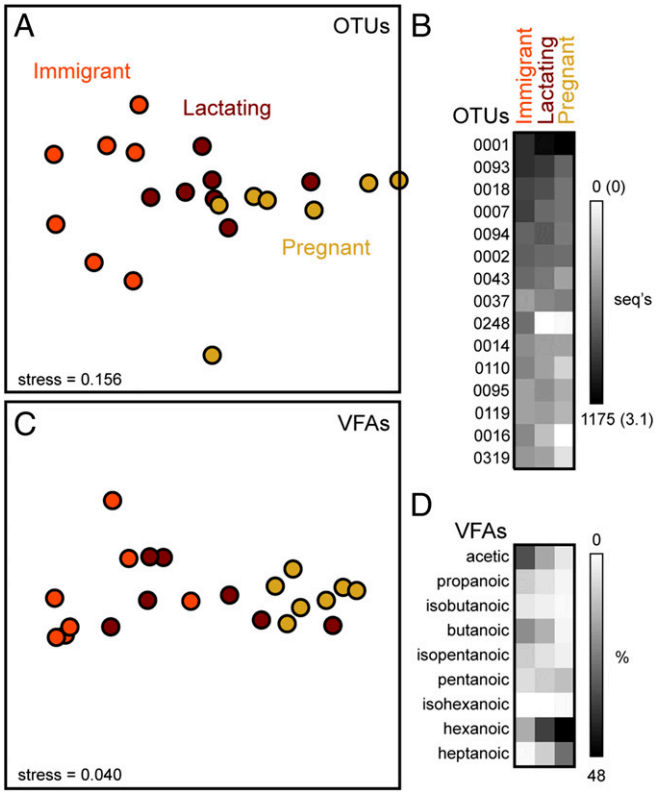

Fig. 2. Variation in the bacterial (OTU) and VFA profiles of the pastes of immigrant male, lactating female, and pregnant female spotted hyenas in the Talek clan. (*A*) An nMDS plot showing variation in the structure (Bray–Curtis index) of paste bacterial communities among Talek clan members. (*B*) A heat map of the mean abundances (out of 1,600 sequences) of the prominent (i.e., top 15 based on average abundance) OTUs in the pastes of Talek hyenas. These data were log-transformed before plotting (values in parentheses). (*C*) An nMDS plot showing variation in the structure of paste VFA profiles in Talek hyenas. (*D*) A heat map of the mean percent abundances of VFAs in the pastes of Talek hyenas. Seven hyenas were sampled from each reproductive class.

differed between the two hyena species (Jaccard index, ANOSIM; MMNR vs. Laikipia: $R = 1.0$, $P = 0.0001$; MMNR vs. Shompole: $R = 1.0$, $P = 0.0001$; Fig. 1*B* and Table S2) (26, 27). The great majority of bacteria (>95% of sequences) in the pastes of both hyena species were members of the order Clostridiales—fermentative anaerobes—in the phylum Firmicutes (28). Less than 1% of Clostridiales sequences in striped hyena pastes were assigned to previously characterized genera (Table S2). In contrast, the Clostridiales in spotted hyena pastes were primarily members of the genera *Anaerococcus*, *Clostridium*, *Fastidiosipila*, *Finegoldia*, *Murdochiella*, *Peptoniphilus*, and *Tissierella*. Spotted hyena pastes also consistently contained fermentative genera outside the phylum Firmicutes, such as *Corynebacterium*, *Propionibacterium* (both Actinobacteria), *Porphyromonas* (Bacteroidetes), and *Fusobacterium* (Fusobacteria) (28–33). At an OTU level, only 11 of 461 OTUs were shared between the pastes of adult spotted hyenas and those of adult striped hyenas from at least one population. Two were prominent (i.e., among the top 15 based on average sequence abundance) members of bacterial communities in striped hyena paste (OTUs 0003 and 0180), but none were prominent in spotted hyena pastes.

Importantly, microbial biogeography alone cannot explain the observed differences in the membership of the paste bacterial communities in adult spotted and striped hyenas. First, although they were not present in the pastes of adult striped hyenas, 13 of the 15 top OTUs in the pastes of MMNR spotted hyenas were present in the pastes of at least one juvenile striped hyena from Laikipia or Shompole, indicating that these OTUs were not restricted geographically to the MMNR. Second, the bacterial communities in the pastes of two spotted hyenas sampled serendipitously in Shompole clustered with bacterial communities from MMNR spotted hyenas rather than with those from sympatric striped hyenas (Fig. S3).

In addition to containing disparate bacterial communities, the pastes of adult spotted and striped hyenas had markedly differently VFA profiles (ANOSIM; MMNR vs. Laikipia: $R = 0.770$, $P = 0.0001$; MMNR vs. Shompole: $R = 0.727$, $P = 0.0001$; Fig. 1 *C* and *D*). The VFA profiles of spotted hyena pastes differed from those of striped hyenas in Laikipia and Shompole in nearly identical ways (Dataset S1 *B* and *C*), again indicating that biogeography is not a primary factor in species differences. The pastes of spotted hyenas had higher percentages of acetic, propanoic, butanoic, pentanoic, and hexanoic acids than those of striped hyenas, whereas the pastes of striped hyenas had much higher percentages of isopentanoic acid (Fig. 1*D* and Dataset S1 *B* and *C*). As with bacterial profiles, there was much greater variation in the VFA profiles of the pastes of spotted than striped hyenas (PERMDISP; MMNR (0.268 ± 0.106) vs. Laikipia (0.114 ± 0.029): $P = 0.0001$; MMNR vs. Shompole (0.096 ± 0.060): $P = 0.0001$; Fig. 1*C*).

The Bacterial and VFA Profiles of Spotted Hyena Pastes Covary, and Within a Hyena Clan Both Profiles Vary with Sex and Reproductive State. The bacterial and VFA profiles of paste covaried among spotted hyenas from the general MMNR population (Mantel test, $R = 0.437$, $P = 0.0002$), and the Talek clan ($R = 0.565$, $P = 0.0001$). There also was strong covariance between the OTU–VFA correlation matrices of MMNR and Talek pastes (correlation *r*, Mantel test, $R = 0.721$, $P = 0.0003$; analysis of 10 shared prominent OTUs; Fig. S4), indicating that the abundance of specific OTUs correlated with the relative abundance of specific VFAs in similar ways at population and clan levels.

At the population level, there was a tendency for the bacterial profiles of paste to differ between male and female spotted hyenas (ANOSIM, $R = 0.113$, $P = 0.0792$), but there was not a consistent effect of sex on the VFA profiles of paste ($R = -0.011$, $P = 0.4427$). Still, the bacterial and VFA profiles of paste did covary among both MMNR males (Mantel test, $R = 0.393$, $P = 0.0397$) and females ($R = 0.400$, $P = 0.0231$).

Among members of the Talek clan, there were pronounced effects of both sex and female reproductive state on the bacterial profiles of paste (Fig. 2 *A* and *B*, Table 1, and Dataset S1 *D* and *F*). The bacterial communities in the pastes of Talek males, lactating females, and pregnant females contained similar numbers of OTUs (Chao1 index, ANOVA, $F_{2,12} = 2.033$, $P = 0.1600$; Table S1). However, bacterial communities in the pastes of males and lactating females were more even than those associated with pregnant females (Simpson index, ANOVA, $F_{2,18} = 8.876$, $P = 0.0021$; male vs. pregnant female: Tukey's test, $Q = 5.90$, $P = 0.0017$; lactating female vs. pregnant female: $Q = 3.67$, $P = 0.046$; Table S1), largely because the bacterial communities in the pastes of pregnant females were dominated by members of OTU 0001 (Dataset S1 *D* and *F*). Overall, there were 343 OTUs in the pastes of Talek hyenas; 120 OTUs were exclusive to Talek males, 54 were exclusive to lactating females, and 46 were exclusive to pregnant females. Few exclusive OTUs were widespread among their respective host class, and those that were widespread were minor members of their communities. In general, Talek males, lactating females, and pregnant females shared the prominent members of their paste bacterial communities, but the relative abundances of these members varied with host sex and reproductive state (Fig. 2*B*, Table 1, and Dataset S1 *D* and *F*).

The VFA profiles of paste also varied among Talek males, lactating females, and pregnant females (Fig. 2 *C* and *D*, Table 1, and Dataset S1 *E* and *F*). The pastes of females, especially pregnant ones, had higher percentages of pentanoic, hexanoic, and heptanoic acids, whereas the pastes of Talek males

Table 1. Nonparametric multivariate analyses of variance confirming that the bacterial (OTU) and VFA profiles of the pastes of Talek immigrant males, lactating females, and pregnant females vary

Composition of paste OTU profiles (Jaccard index)	
Global effect	$F = 2.468$, $P = 0.0001$
Male vs.lactating female	$F = 2.485$, $P = 0.0011$
Male vs.pregnant female	$F = 3.070$, $P = 0.0020$
Lactating vs. pregnant female	$F = 1.757$, $P = 0.0177$
Structure of paste OTU profiles (Bray–Curtis index)	
Global effect	$F = 4.181$, $P = 0.0001$
Male vs.lactating female	$F = 3.789$, $P = 0.0006$
Male vs.pregnant female	$F = 6.023$, $P = 0.0007$
Lactating vs. pregnant female	$F = 2.411$, $P = 0.0398$
Structure of paste VFA profiles (Bray–Curtis index)	
Global effect	$F = 16.23$, $P = 0.0001$
Male vs.lactating female	$F = 5.779$, $P = 0.0116$
Male vs.pregnant female	$F = 43.81$, $P = 0.0008$
Lactating vs. pregnant female	$F = 9.209$, $P = 0.0069$

Seven hyenas were sampled from each reproductive class.

had higher percentages of acetic, propanoic, isobutanoic, and butanoic acids. Notably, the structures of the bacterial and VFA profiles of paste covaried among Talek's lactating (Mantel test, $R = 0.7641$, $P = 0.0001$) and pregnant ($R = 0.3768$, $P = 0.0468$) females, and tended to covary among Talek's males ($R = 0.3077$, $P = 0.0766$).

The Bacterial and VFA Profiles of Striped Hyena Pastes Covary. The bacterial and VFA profiles of striped hyena pastes covaried (Mantel test, $R = 0.7697$, $P = 0.0001$), and this covariance was evident among pastes from both Laikipia ($R = 0.7176$, $P = 0.0028$) and Shompole ($R = 0.7952$, $P = 0.0002$). Furthermore, the OTU–VFA correlation matrices for Laikipia and Shompole covaried (correlation r, Mantel test, $R = 0.2341$, $P = 0.0461$; Fig. S4), indicating that the abundance of specific prominent OTUs correlated with the percent abundance of specific VFAs in similar ways in the pastes of striped hyenas from the two different populations.

The structure of paste bacterial communities differed between the Laikipia and Shompole populations (ANOSIM, $R = 0.5716$, $P = 0.0001$; Fig. 3 *A* and *B* and Dataset S1 *G* and *H*). Variation in bacterial community structure was very low within each population, especially among adults (Fig. 3*A* and Dataset S1*H*). Membership of paste bacterial communities also differed between the Laikipia and Shompole populations (Jaccard index, ANOSIM, $R = 0.5747$, $P = 0.0001$). There were 443 OTUs in the pastes of striped hyenas; 59 were found exclusively in the Laikipia population, and 314 were found exclusively in the Shompole population. However, very few of the exclusive OTUs (2/59, 4/314) were found in more than half of the pastes from hyenas in the respective populations. In general, striped hyenas in Laikipia and Shompole shared the prominent members of their paste bacterial communities, but the relative abundances of these members differed between the two populations (Fig. 3*B* and Dataset S1 *G* and *H*). As a potential consequence, there was a tendency for paste VFA profiles to differ between the Laikipia and Shompole populations as well (ANOSIM, $R = 0.1091$, $P = 0.0508$; Fig. 3 *C* and *D*, Dataset S1 *G* and *H*).

Controlled for source population, there was a modest effect of sex on the structure of bacterial communities in the pastes of striped hyenas (two-way ANOSIM; population: $R = 0.5563$, $P = 0.0001$; sex: $R = 0.1170$, $P = 0.0382$) but not on paste VFA profiles (population: $R = 0.0942$, $P = 0.0933$; sex: $R = 0.0281$, $P = 0.2712$). However, when controlled for source population, the bacterial and VFA profiles of pastes did covary among male and female striped hyenas (partial Mantel test; male: $R = 0.8446$, $P = 0.0001$; female: $R = 0.7397$, $P = 0.0002$).

Discussion

We tested first predictions of the fermentation hypothesis for chemical communication in hyenas and showed that (*i*) hyena pastes contained fermentative bacteria, (*ii*) the bacterial and VFA profiles of hyena pastes covaried, and (*iii*) these profiles differed between hyena species and, within a spotted hyena clan, they varied with sex and reproductive state.

The bacteria in hyena pastes belong to clades of fermentative bacteria whose metabolisms yield varying amounts of acetic, propanoic, isobutanoic, butanoic, isopentanoic, isohexanoic, and hexanoic acids (28–33). These differences suggest that variation in the structure of paste bacterial communities should result in variation in the structure of paste VFA profiles. Here we found that the structures of the bacterial and VFA profiles of hyena pastes strongly covaried. Prior studies using microbial fingerprinting surveys revealed that the bacterial and odor profiles of human axillae covaried among people who adhered to presampling guidelines on bathing and deodorant use (34) and that the bacterial and odor profiles of the urine-marks of laboratory mouse strains, *Mus domesticus*, were partially correlated (35). The current study focused on wild populations of mammals, and showed strong, consistent covariance between the bacterial and odor profiles of mammalian scent gland secretions. The study also demonstrated

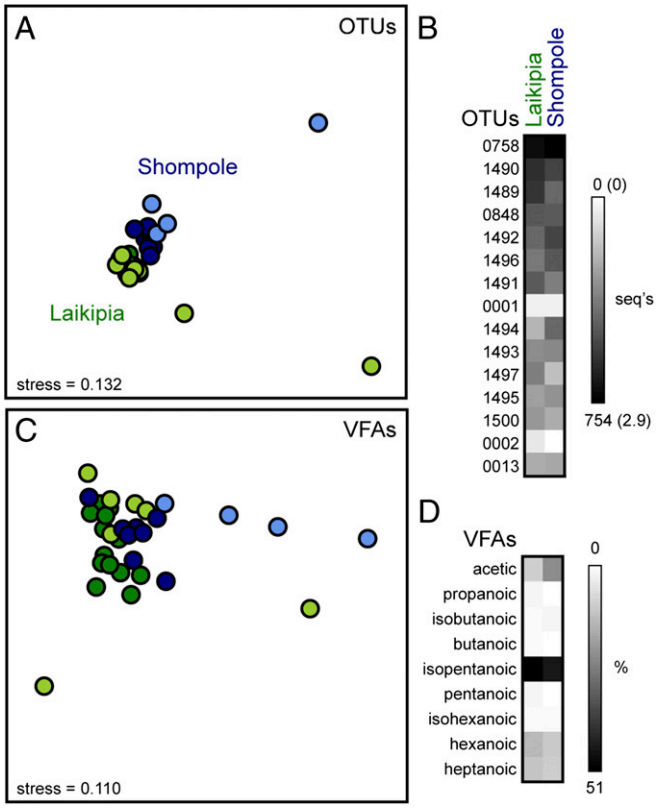

Fig. 3. Differences in the bacterial (OTU) and VFA profiles of the pastes of striped hyenas in Laikipia and Shompole. (*A*) An nMDS plot showing a difference between the structure (Bray–Curtis index) of paste bacterial communities in the Laikipia and Shompole populations. Lighter shading denotes samples obtained from juveniles. (*B*) A heat map of the mean abundances (out of 1,600 sequences) of the prominent (i.e., top 15 based on average abundance) OTUs in the pastes of striped hyenas. These data were log-transformed before plotting (values in parentheses). (*C*) An nMDS plot of the structures of paste VFA profiles in Laikipia and Shompole. (*D*) A heat map of the mean percent abundances of VFAs in the pastes of striped hyenas from the two populations. Twenty striped hyenas were sampled from Laikipia (8 male/12 female), and 13 (six male/ seven female) were sampled from Shompole.

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that the two profiles covaried not only across but also within species, sex, and, in the spotted hyena, reproductive classes.

There were robust effects of species identity on the bacterial and VFA profiles of hyena paste. Spotted and striped hyena pastes contained each of the VFAs studied here, but they were present in markedly different proportions. The two hyenas are sympatric in areas of West and East Africa, including Kenya (36), so this finding is consistent with it being advantageous for sympatric taxa to distinguish readily between the homologous signals of conspecifics and heterospecifics (37). Interestingly, the structures of spotted and striped hyena paste VFA profiles also differed in their degree of intraspecific variation, with variation among spotted hyena paste VFA profiles far exceeding the variation among striped hyena pastes. Given that spotted hyenas are highly social and striped hyenas are largely solitary, this finding is consistent with the social complexity hypothesis for animal signaling, which posits that frequent interactions in various contexts with many different individuals results in the evolution of more complex signaling systems (38, 39).

At the clan level, there was a pronounced effect of sex on both the bacterial and VFA profiles of spotted hyena paste. A prior study of a captive colony of spotted hyenas showed that they discriminated readily between the pastes of males and females in the colony, indicating that paste odor profiles, on a local scale, were sex specific (25). A different, population-level study of spotted hyenas in the Serengeti National Park, Tanzania, did not find evidence that sex affected paste odor profiles (24). We also did not find a consistent effect of sex on the bacterial or VFA profiles of spotted hyena paste at the population level. The bacterial and odor profiles of spotted hyena pastes are generally clan specific (15, 16). The purported mechanism for clan-specific paste odors is that clan members develop more homogeneous paste bacterial communities than the general population through cross-infection fostered by consistently overmarking the same pasting sites (12, 15, 16, 40). Indeed, in this study, the structures of bacterial communities in the pastes of spotted hyena males and lactating females were more variable in the general MMNR population than in the Talek clan [PERMDISP; MMNR (0.387 ± 0.092) vs. Talek (0.292 ± 0.059), $P = 0.0017$]. Collectively, these data suggest that social interactions at the clan level (e.g., sex-specific patterns in overmarking) may confound population-level analyses. Among striped hyenas, there was an effect of sex on the bacterial but not the VFA profiles of paste. This finding suggests that, in the striped hyena, sex may not be communicated through paste or that it is communicated via volatiles other than those studied here.

The reproductive state of female spotted hyenas substantially affected the structures of paste bacterial and VFA profiles. Pregnancy can dramatically alter mammalian oral, vaginal, and gut bacterial communities (41–43). Here we show that pregnancy can alter the microbiota of specialized signaling organs as well. During pregnancy, spotted hyena females have elevated levels of testosterone and estrogen (44, 45). It is well established that steroid hormones are present in mammalian sebaceous and apocrine glands (i.e., the machinery of scent glands) and that they affect gland morphology, production, and chemistry (6, 46–48). It appears they also may affect the structures of bacterial communities in these glands in ways that could signal the reproductive state of female hosts effectively.

This study provides strong empirical support for the fermentation hypothesis for chemical communication in hyenas. However, two further predictions need to be tested. First, if symbiotic bacteria are the source of paste VFAs, then, if provided with appropriate growth conditions, paste cultivars should produce, to varying degrees, the VFAs studied here. Alternatively, their genomes should contain genes coding for the fermentation pathways leading to the production of these VFAs. Second, if the variation we found in paste VFA profiles has signaling relevance, then hyenas should discriminate among synthetic mixtures of these volatiles representing samples from this study. Importantly, we explicitly tested the fermentation hypothesis for chemical communication because it was proposed nearly 40 y ago to explain the scent marking systems of mammals (11, 12), and many of the volatile components of hyena paste are known products of bacterial fermentation (23, 24). However, extrapolated to a general symbiotic hypothesis for animal chemical communication, the fermentation hypothesis accommodates other odorous microbial metabolites (e.g., longer-chain fatty acids and their esters), symbiotic microbes (e.g., fungi and archaea), signaled host characteristics (e.g., genotype, health, and social status), and animal classes. In fact, the explanatory potential of this hypothesis is limited only by the capacity of hosts' social and physiological circumstances to alter the structure of their symbiotic microbial communities in ways that consequently affect hosts' odor profiles in signaling-relevant ways (6). Therefore, evaluating the potential of the symbiotic hypothesis for animal chemical communication will be a critical step in elucidating the contributions of symbiotic microbes to animal behavior.

Methods

Collection of Scent Gland Secretions (Pastes). Pastes were collected directly from the subcaudal scent pouches of anesthetized (*SI Methods*) spotted hyenas in the MMNR (1994–2008) and striped hyenas in the Laikipia District (2001–2003) and at the Shompole and Olkirimatian Group Ranches (2007–2009; hereafter, "Shompole"), Kenya (Fig. S1). 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 their bacterial and VFA profiles were determined (Table S3).

The 40 sampled spotted hyenas resided in the north-central region of the MMNR. They represented the general MMNR population (nine males/10 females from >10 clans) as well as a single, intensively studied clan (seven males/seven lactating females/seven pregnant females from the Talek clan). Only one individual, CNLF428, appeared in our prior study (15). Females from the general MMNR population were lactating when sampled. Talek lactating females did not give birth in the 150 d after they were sampled and therefore were, to the best of our knowledge, not pregnant [110 d mean gestation time (19)]. Talek pregnancies were confirmed via ultrasound imaging of fetuses and/or by the female giving birth within 80 d of being sampled. Juvenile spotted hyenas were not included in this study because they do not consistently produce appreciable amounts of paste (40). Paste samples were serendipitously obtained from two spotted hyenas in Shompole. These samples were large enough for us to characterize their bacterial but not their VFA profiles, so they were included only in a single supplemental analysis (Fig. S3). The 33 sampled striped hyenas represented the general populations of the north-central Laikipia District (eight males/12 females) and Shompole (six males/seven females). They included both adults (22) and juveniles (11) and constituted all the individual striped hyenas for which paste samples were sufficiently large for bacterial and VFA analysis. Reproductive data were not available for adult female striped hyenas.

Bacterial (OTU) Surveys of Pastes. DNA was extracted from paste sample aliquots (~0.05 g) using a MO BIO UltraClean fecal DNA kit. Bacterial 16S rRNA genes in extractions were PCR amplified (*SI Methods*) using two broadly conserved, degenerate primers targeting the V6–V4 variable regions of the 16S gene (1046R: 5′–CGACRRCCATGCANCACCT–3′; 518F: 5′–CCAGCAGCYGCGGTAAN–3′). Nucleotide sequencing was performed on 454 GS FLX Titanium and GS Junior instruments at the Marine Biological Laboratory in Woods Hole, MA, and at Michigan State University. Postsequencing, 454 run files were processed using mothur software (v. 1.27.0; *SI Methods*) (49). Sequences were binned into OTUs based on a 97% sequence similarity. Each paste bacterial community then was iteratively subsampled 15 times to the depth of the least-represented sample (1,600 sequences), and the mean abundances of individual OTUs per sample were calculated and rounded to the nearest whole number. The final data set contained 865 OTUs (471 singletons). Representative sequences of these OTUs and associated metadata are available in GenBank (accession nos. KC705471–KC706325) and Dataset S2.

VFA Surveys of Paste. Branched and linear VFAs were extracted from aliquots (0.025 g) of paste using methyl tert-butyl ether as solvent. Specifically, we targeted acetic, propanoic, isobutanoic, butanoic, isopentanoic, pentanoic, isohexanoic, hexanoic, and heptanoic acids. Samples were analyzed using an Agilent Technologies' 6890N/5973 inert GC/MS system equipped with a 30-m DB-wax column (250-μm inner diameter × 0.25-μm film thickness). One microliter of the sample was injected using an Agilent 7683 auto-injector. Two blanks consisting of the solvent mixture spiked

with 5 μL 85% (wt/vol) formic acid were included between each sample to prevent any carry-over. The percent peak abundances of VFAs for each sample were determined using QuanLynx software (Micromass). Detailed protocols for VFA extraction and GC/MS analyses, including the use of internal and external standards, are provided in *SI Methods*.

Characterization of the Similarities of the OTU and VFA Profiles of Paste. Before analyses, OTU abundance data were $\log_{10}$ (x +1) transformed to temper the contributions of highly prominent OTUs to quantitative similarity index calculations (50). Quantitative (i.e., structural) similarities of the OTU or VFA profiles of paste samples were characterized using the Bray–Curtis similarity index (51). Unless otherwise noted, multivariate statistical tests were based on the Bray–Curtis index. Qualitative (i.e., compositional) similarities of the OTU profiles of pastes were characterized using the Jaccard similarity index (51). A detailed discussion of graphical and statistical data analysis is provided in *SI Methods*.

Animal Use and Care. Our research, described in Animal Research Protocol Institutional Animal Care and Use Committee (IACUC) 05/11–110-00, was approved most recently on June 15, 2012 by the IACUC at Michigan State University and complies with Kenyan Law.

ACKNOWLEDGMENTS. We thank the Kenyan Ministry of Education, Science and Technology, the Kenyan Wildlife Service, the Narok County Council, the Senior Warden of the Masai Mara National Reserve, the Loisaba, Mpala, and Kisima Ranches (Laikipia), and the Shompole and Olkirimatian Group Ranches for permissions and support of this research. We thank Christine Drea (Duke University), Hilary Morrison (Marine Biological Laboratory), and Dan Jones, Scott Smith, Abby Vanderberg, Kylie Farrell, and Alex Schmidt (Michigan State University) for contributing their technical expertise. This study was funded by National Science Foundation and BEACON Center for the Study of Evolution in Action Grants IOS0920505, IOS0819437, IOS0618022, IOS1121474, and DBI0939454.

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