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Research Article

RESEARCH ARTICLE

Body site-specific microbiota reflect sex and age-class among wild spotted hyenas

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One sentence summary: This study comprehensively examines the microbiota of a terrestrial carnivore at multiple body sites and reports that the microbiota varied considerably among body sites, exhibited strong specificity among individuals and its variation was associated with host sex and age-class.

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ABSTRACT

Host-associated microbial communities, henceforth 'microbiota', can affect the physiology and behavior of their hosts. In mammals, host ecological, social and environmental variables are associated with variation in microbial communities. Within individuals in a given mammalian species, the microbiota also partitions by body site. Here, we build on this work and sequence the bacterial 16S rRNA gene to profile the microbiota at six distinct body sites (ear, nasal and oral cavities, prepuce, rectum and anal scent gland) in a population of wild spotted hyenas (*Crocuta crocuta*), which are highly social, large African carnivores. We inquired whether microbiota at these body sites vary with host sex or social rank among juvenile hyenas, and whether they differ between juvenile females and adult females. We found that the scent gland microbiota differed between juvenile males and juvenile females, whereas the prepuce and rectal microbiota differed between adult females and juvenile females. Social rank, however, was not a significant predictor of microbiota profiles. Additionally, the microbiota varied considerably among the six sampled body sites and exhibited strong specificity among individual hyenas. Thus, our findings suggest that site-specific niche selection is a primary driver of microbiota structure in mammals, but endogenous host factors may also be influential.

Keywords: microbiota; microbiome; spotted hyenas; 16S rRNA gene sequencing; host–microbe interactions; body sites

INTRODUCTION

Animal bodies are home to structurally and functionally organized microbial communities, termed 'microbiota', which can strongly affect their host's physiology, behavior and fitness (Archie and Theis 2011; Archie and Tung 2015; Vuong *et al.* 2017). For example, in the gastrointestinal tracts of folivorous and myrmecophagous mammals (Delsuc *et al.* 2014; Alfano *et al.* 2015), resident microbes can convert tough compounds (i.e. cellulose and chitin) into readily available nutrients and energy for

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their host. In multiple species of carnivores (Theis *et al.* 2013; Leclaire, Nielsen and Drea 2014; Buesching *et al.* 2016; Leclaire *et al.* 2017), microbiota inhabiting scent gland secretions co-vary with the gland's odorous biochemical profiles and include odor-producing bacteria, indicating that microbes likely contribute to their host's chemical signals. Furthermore, in insectivorous bats, bacteria from the skin exhibit antifungal properties against the pathogen that causes white-nose syndrome, implicating these microbes in the pathogen defenses of their hosts (Hoyt *et al.* 2015; Hamm *et al.* 2017; Lemieux-Labonté *et al.* 2017). Thus, resident microbes and their genomes, collectively referred to as the 'microbiome', are functionally important in shaping the phenotypes of their hosts. Hence, identifying the environmental and host factors that affect variation in the microbiota and microbiome, and asking how this may affect host phenotype, are key lines of inquiry in host–microbial ecology (McFall-Ngai *et al.* 2013; Bordenstein and Theis 2015; Antwis *et al.* 2017).

Numerous environmental, social and physiological host factors are associated with variation in the microbiota within and among mammalian host species. Among the key drivers of variation in the mammalian microbiota, particularly the gut microbiota, are host diet and phylogeny (e.g. Ley *et al.* 2008; Groussin *et al.* 2017; Muegge *et al.* 2017; Li *et al.* 2018a; Youngblut *et al.* 2019). The microbiota might also be sensitive to the host's social and ecological environment, as some variation in the microbiota can be attributed to their host's social interactions, season, habitat and geography (e.g. Tung *et al.* 2015; Sommer *et al.* 2016; Perofsky *et al.* 2017; Greene *et al.* 2019b; Watson *et al.* 2019). Lastly, microbiota are also known to vary with physiological and host-specific factors such as genetic variation, sex, age and individual identity (e.g. Leclaire, Nielsen and Drea 2014; Blekhman *et al.* 2015; Theis *et al.* 2016; Cuscó *et al.* 2017a; Grosser *et al.* 2019). Within individual hosts, however, microbiota are often strongly structured by anatomical body region. In a range of mammalian hosts, including humans, primates, marine mammals, marsupials, bats, carnivores and domestic animals, the microbiota vary among body sites (e.g. Huttenhower *et al.* 2012; Carvalho *et al.* 2014; Alfano *et al.* 2015; Cheng *et al.* 2015; Bik *et al.* 2016; Dietrich *et al.* 2017; Dietrich *et al.* 2018; Rothschild *et al.* 2018; Strube *et al.* 2018). Host-associated microbial communities may also vary within specific regions of a body site, as has been demonstrated for the mammalian gastrointestinal tract (e.g. stomach, small intestine, cecum, large intestine, feces; Dougal *et al.* 2012; Li *et al.* 2017a; Greene and Mckenney 2018; He *et al.* 2018b), the skin (e.g. axillae, nose, ears, digits, limbs; Hoffmann *et al.* 2014; Cuscó *et al.* 2017a; Kamus, Theoret and Costa 2018) and the oral cavity (e.g. gums, molars, plaque; Huttenhower *et al.* 2012).

Here, we build upon this prior work by using 16S rRNA gene sequencing to characterize the diversity and structure of host-associated microbiota at six distinct body sites in a gregarious large carnivore, the spotted hyena (*Crocuta crocuta*). Spotted hyenas inhabit much of sub-Saharan Africa (Mills and Hofer 1998) and live in social groups, called 'clans'. Clans may contain over 90 individuals, and usually consist of multiple overlapping generations of natal females and their offspring, along with a few immigrant males. Their societies are structured by linear dominance hierarchies, in which an individual's position within the hierarchy determines its priority of access to resources (Kruuk 1972; Frank 1986). Hyena societies are also characterized by female dominance, male-biased dispersal and a high degree of fission–fusion dynamics, such that individuals move among subgroups several times per day (Kruuk 1972; Frank 1986; Höner *et al.* 2007; Smith *et al.* 2008). Hyenas are matrilineal, and each new offspring inherits the rank immediately below that of its mother but above those of its older siblings (Holekamp and Smale 1993; Smale, Frank and Holekamp 1993). Spotted hyenas bear litters of one or two cubs, which are reared at communal dens for the first 9 to 12 months of life; they are weaned at 12–18 months, and reach reproductive maturity at 24 months, although most females do not bear offspring of their own until they are at least 36 months of age (Hofer and East 1995; Holekamp, Smale and Szykman 1996). To communicate, spotted hyenas utilize signals via multiple sensory modalities, including a rich repertoire of vocalizations (Gersick *et al.* 2015) and odorous secretions from their scent glands (Woodmansee *et al.* 1991; Drea *et al.* 2002; Burgener *et al.* 2008; Theis, Schmidt and Holekamp 2012; East, Burgener and Hofer 2013; Theis *et al.* 2013).

Specifically, in this study, we inquire whether body site specificity of the microbiota is observed in adult and juvenile hyenas. We also evaluate whether these bacterial communities vary with host sex or social rank among juvenile hyenas. Lastly, we investigate whether the microbiota differs between juvenile females and adult females at each body site. Prior research on spotted hyenas has shown that the anal scent gland microbiota varies with host sex, social group and reproductive state (Theis *et al.* 2013), and that gut microbiota diversity varies with host age (Heitlinger *et al.* 2017). However, we know little about the microbiota occupying other hyena body regions. Thus, our study will help establish a baseline understanding of the microbiota occupying six anatomical body sites of a large carnivore species in its natural habitat.

METHODS

Behavioral and demographic data collection

We identified individual hyenas by their unique spot patterns, determined their sex based on phallic morphology (Frank, Glickman and Powch 1990) and calculated birthdates to ±7 days based on the appearance of cubs when first observed (Holekamp, Smale and Szykman 1996). We defined juveniles as hyenas <24 months old, and older animals were considered to be adults. Hyenas were assigned a dominance rank based on their position in a matrix ordered by submissive behaviors displayed during dyadic agonistic encounters (Strauss and Holekamp 2019). In our analyses, social rank was normalized (to values between 0 and 1), such that the highest ranking hyena had a value of 1 and the lowest ranking hyena had a value of 0. Juveniles were assigned the same ranks as their mothers. Our statistical power was insufficient to test whether the microbiota of adult females varied with clan membership or social rank, so these samples were pooled and used only when determining whether the microbiota varied among body sites or age-classes. Sample metadata are provided as supplementary materials (Supplemental file 1: Sample metadata, Supporting Information).

Sample collection

Bacterial swabs were collected from 12 adult and 24 juvenile spotted hyenas inhabiting the Massai Mara National Reserve, Kenya, between May 2012 and July 2014. The adult hyenas were all females, and represented the Talek ($N = 5$), Fig Tree ($N = 3$) and Serena South ($N = 4$) clans within the Reserve. The juveniles included 13 females and 11 males from the Talek clan. The hyenas were anesthetized with Telazol (6.5 mg/kg), and swabs were obtained from the anal scent gland, rectum, prepuce, oral cavity (gum line above the upper 3rd pre-molar), nares and ear. Swabs were stored in cryogenic vials in liquid nitrogen until transport

to Michigan State University, where they were stored at −80°C until DNA extraction.

DNA extractions and 16S rRNA gene sequencing

DNA was extracted from bacterial swabs using PowerSoil DNA Isolation Kits (MOBIO Laboratories, Inc., Carlsbad, CA), following the manufacturer's recommended protocol, with two minor modifications. For each sample, we removed 200 μL bead solution and replaced it with 200 μL phenol:chloroform:isoamyl alcohol (25:24:1, v/v; Thermo Scientific, Waltham, MA). We also incubated the swab in bead solution within the bead tube for 10 min, and vortexed the bead tube for 1 min before removing the swab aseptically and resuming the DNA extraction protocol. These modifications were implemented to increase the DNA yield of our low-biomass samples. The order of DNA extractions was randomized to minimize sampling bias (i.e. extracting samples from only one body site or from the same individual hyena). We also completed six blank DNA extraction kit controls (i.e. DNA extractions of sterile swabs) to control for potential background DNA contamination (PowerSoil DNA Isolation Kits, MOBIO Laboratories, CA). The V4 region of the bacterial 16S rRNA gene was targeted for paired-end sequencing (~253 bp per sequence) on the Illumina MiSeq platform at the Michigan State University Genomics Core (East Lansing, MI). Sample preparation, nucleotide sequencing and preliminary quality filtering were completed as described previously (Caporaso *et al.* 2012; Kozich *et al.* 2013).

Sequence processing

All sequence processing was conducted using mothur software (v.1.36.1; Schloss *et al.* 2009), following the MiSeq standard operating procedure (https://www.mothur.org/wiki/MiSeq_SOP). Briefly, forward and reverse reads were joined into contigs, generating a total of 11 331 400 paired-end sequences. After initial quality filtering, the remaining sequences (8745 743) were aligned to the Silva reference database (v.4; Quast *et al.* 2013). Chimeric sequences were detected and removed using the UCHIME algorithm (Edgar *et al.* 2011), and the remaining sequences were taxonomically classified using the Ribosomal Database Project reference files (RDP; v.9; Cole *et al.* 2014). Sequences deemed to have come from Chloroplasts, Mitochondria, Archaea or Eukarya were filtered from our dataset, leaving 8363 519 total sequences, which were clustered *de novo* into operational taxonomic units (OTUs) at 97% nucleotide similarity (Westcott and Schloss 2017).

A total of 16 OTUs had an average relative abundance of >1% across our blank DNA extraction kit controls (for the list of OTUs, see Supplemental file 2: Table S1, Supporting Information); most were previously documented contaminants of DNA extraction kits and/or reagents (Salter *et al.* 2014; Glassing *et al.* 2016). We removed these OTUs from the dataset, with the exception of *Providencia* (OTU0006). *Providencia* was kept because it had an average relative abundance >1% among both biological and technical control samples, and members of this genus are common residents of the mammalian gastrointestinal tract (Manos and Belas 2006; Li *et al.* 2014; Yadav, Verma and Chauhan 2018). We subsampled individual samples to 13 340 sequence reads/samples prior to analysis to avoid biases due to sequencing effort. This subsampling cutoff was the third-lowest number of sequences found in our dataset and was selected because it satisfied saturation for the majority of our samples and minimized data loss (i.e. a higher cutoff would have resulted in additional samples excluded from statistical analyses). The table of OTUs and their associated taxonomic classifications can be found in Supplemental file 3: OTU table and Supplemental file 4: OTU Taxonomy (Supporting Information).

Microbial community composition analyses

To visualize microbiota composition, we constructed heat maps and stacked bar plots in R v.3.4.3 (R Core Team 2017). For the raw relative abundances of each bacterial taxon at each body site, see Supplemental file 5: Table S2 (adults) and Supplemental file 6: Table S3 (juveniles) (Supporting Information). We used linear discriminant analysis (LDA) effect size (LEfSe) to identify the taxa that were enriched in particular samples (i.e. in one body site relative to others) using default parameters (Segata *et al.* 2011).

Analyses of alpha-diversity

Microbiota α-diversity was estimated in mothur prior to deleting singletons (sequences observed only once in the dataset) and doubletons (sequences observed only twice). Specifically, community richness was characterized using the Chao1 nonparametric richness estimator, and community evenness was characterized using Shannon diversity and Simpson's diversity indices. Chao 1 values were log-transformed prior to analyses, due to their skewed, high values (max. 5000). The majority of rarefaction curves of OTU richness for a given number of sequences reached saturation, and Good's coverage values of all body sites averaged greater than 95%, indicating that sequencing depth was sufficient for analysis of these communities (Supplemental file 2: Figure S1 and Table S6, Supporting Information). The effect of predictor variables (i.e. age-class, sex, social rank) on each measure of α-diversity was statistically evaluated using linear mixed effects models in R with the lme4 package, specifying hyena identity as a random effect and body site as one of the two fixed variables (e.g. y ~ body site + sex + 1|hyena id; Bates *et al.* 2015). The significance of the effect of each predictor variable on microbiota α-diversity was assessed via Wald Chi Square Tests on the linear mixed effects model using the R car package (Fox, Weisberg and Fox 2011). If a particular main effect was deemed statistically significant ($P < 0.05$), we followed up with multiple comparison testing using the multcomp R package and report Benjamini–Hochberg adjusted *P*-values (Hothorn, Bretz and Westfall 2008).

Analyses of beta-diversity

Prior to analysis of β-diversity, singletons and doubletons were removed from the dataset. For all the β-diversity analyses, we used the vegan package in R (R Core Team 2017; Oksanen *et al.* 2018). β-diversity among samples was assessed using Jaccard (presence/absence data) and Bray–Curtis (relative abundance data) distance measures. To visualize microbiota similarity, we generated principal coordinate analysis (PCoA) plots from the distance matrices and coupled these with permutational multivariate analysis of variance (PERMANOVA) tests. To assess the effect of continuous predictor variables on microbiota similarity (i.e. social rank), we used Mantel tests with Spearman correlations. Lastly, to test for differences in microbiota dispersion (i.e. between-sample variance), we ran permutation tests of multivariate dispersions (PERMDISP2; Anderson 2006). To visualize the degree of dispersion in the microbiota, the output from PERMDISP2 was also plotted in R (R Core Team 2017).

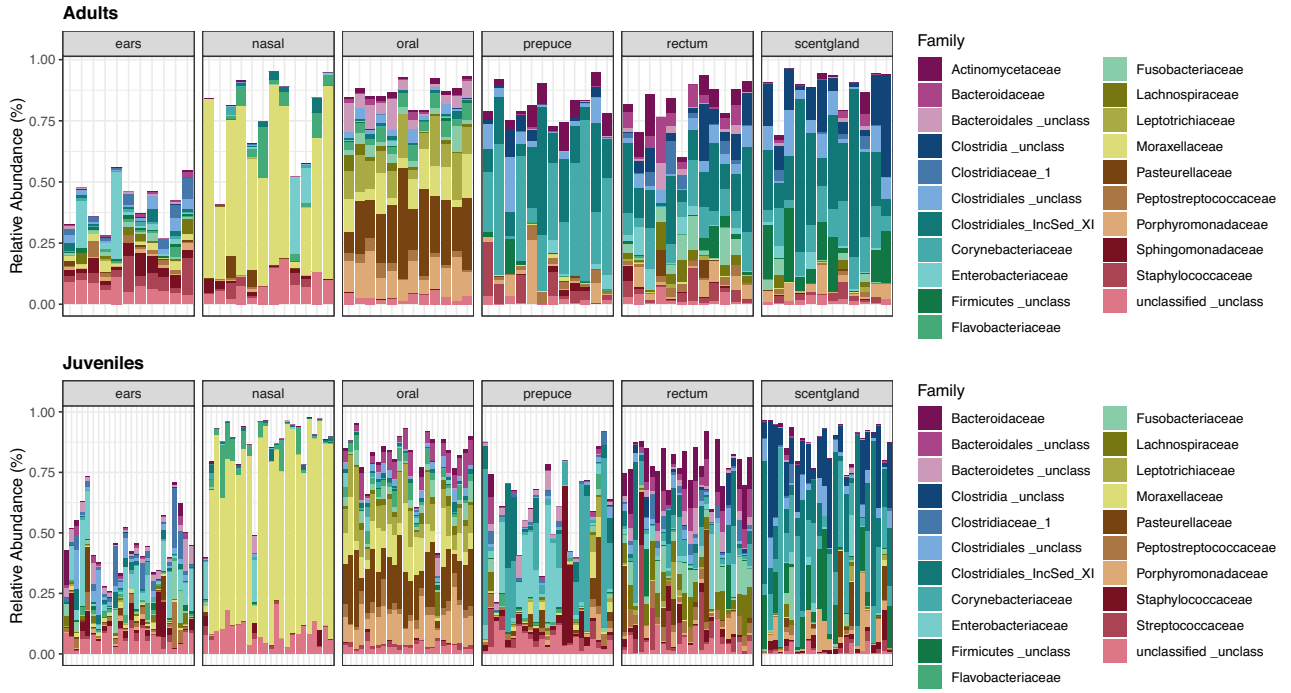

Figure 1. Microbiota composition at multiple body sites in adult and juvenile spotted hyenas. Stacked bar plots showing the relative frequency of 16S rRNA gene sequences assigned to each bacterial family across samples in the ears, nose, mouth, prepuce, rectum and anal scent gland of adults (top) and juveniles (bottom). Each individual bar represents a sample and 1.00 equals 100%. Not all individual bars reach 1.00 because the rest of the taxa were not among the top 21 most abundant. Note how the keys from the two panels are almost identical, except for the names of the first two and last two taxa.

RESULTS

Microbiota composition is body site specific

The microbiota of adult and juvenile spotted hyenas were niche specific, and body sites varied greatly in the relative abundances of their bacterial phyla, families and genera (Fig. 1; Supplemental file 2: Figures S2 and S3, Supporting Information). In adult females, the ear microbiota were not composed of a few dominant taxa, but rather of many taxa found at low abundances (for raw taxa counts, see Supplemental file 5, Supporting Information). The nasal communities, however, contained a single predominant bacterial family, *Moraxellaceae* (51%; Supplemental file 2: Figure S2, Supporting Information). The oral cavity was mostly inhabited by *Pasteurellaceae* (23%), *Leptotrichiaceae* (12%) and *Porphyromonadaceae* (12%), and the rectum by *Clostridiales_XI* (*Anaerococcus*; 19%), *Corynebacteriaceae* (*Corynebacterium*; 9%), unclassified *Clostridia* (6%) *and Bacteroidaceae* (5%). The prepuce and anal scent gland microbiota were similar in composition; both were dominated by *Clostridiales_XI* (mostly *Anaerococcus*; 19% in prepuce; 29% in scent gland) and *Corynebacteriaceae* (*Corynebacterium*; 31% in prepuce; 11% in scent gland). Many of the abundant taxa at each body site were identified by LEfSe as being differentially abundant among body sites, particularly in the ears, nose and mouth (Supplemental file 7: Table S4, Supporting Information).

As was observed in adults, microbiota composition also varied among body sites in juvenile hyenas (Fig. 1; Supplemental file 2: Figures S2 and S3, Supporting Information). Here again, the juvenile ear microbiota were not dominated by a single bacterial type, but the nasal microbiota mostly contained *Moraxellaceae* (68%), and the scent gland harbored high abundances of *Anaerococcus*, *Corynebacterium* and *Clostridia* (20, 19 and 15%, respectively; for raw taxa counts, see Supplemental file 6, Supporting Information). The oral cavity was primarily colonized by *Pasteurellaceae* (18%), *Porphyromonadaceae* (11%), *Leptotrichiaceae* (11%) and *Moraxellaceae* (11%). The juvenile rectum was not dominated by any particular bacterial family, but harbored equal numbers of *Bacteroidaceae*, *Lachnospiraceae*, *Fusobacteriaceae* and *Streptococcaceae* (all ~8%). The juvenile prepuce had high *Corynebacteriaceae* (*Corynebacterium*; *16%*) and *Enterobacteriaceae* (*Providencia*; *10%*) relative abundances. As in adults, many of the aforementioned taxa were among those that LEfSe identified as being differentially abundant among body sites (Supplemental file 7: Table S5, Supporting Information).

Microbiota α-diversity and β-diversity vary among body sites

Body sites also varied in their microbiota richness and evenness in both adult and juvenile hyenas (Table 1; Fig. 2); for means of each diversity index, see Supplemental file 2: Table S6 (Supporting Information). Generally, the microbiota of the ear, mouth and rectum were the most diverse, whereas the preputial, nasal and anal scent gland microbiota were the least diverse (Fig. 2); for post-hoc comparisons, see Supplemental file 2: Tables S7 and S8 (Supporting Information). Furthermore, β-diversity analyses confirmed that microbiota structure also varied among body sites, both when taking into account the relative abundances of taxa (Bray–Curtis PERMANOVA; $R^2 = 0.42$, $P = 0.001$ for adults, $R^2 = 0.37$, $P = 0.001$ for juveniles) and only their presence or absence (Jaccard PERMANOVA; $R^2 = 0.15$, $P = 0.001$ for both adults and juveniles). PCoA ordinations using Bray–Curtis distances showed that the nasal and oral microbiota were different from one another and from those at other body sites (Fig. 3); therefore, we also plotted the remaining body sites separately from the nose and mouth to better visualize their variation (Fig. 4). This

Table 1. Body sites vary in their microbiota richness and evenness (α-diversity).

Predictor	α-Diversity metric	DF	χ^2	*P*-value
Body site (12 adults; 6 sites; 71 samples)	Chao 1 richness	5	12.99	0.023*
	Shannon diversity index	5	68.95	1.69×10^{-13}***
	Simpson's index (1D)	5	66.49	5.4×10^{-13}***
Body site (24 juveniles; 6 sites; 143 samples)	Chao 1 richness	5	39.04	2.32×10^{-7}***
	Shannon diversity index	5	258.9	$<2.2 \times 10^{-16}$***
	Simpson's index (1D)	5	127.83	$<2.2 \times 10^{-16}$***

Shown are the Chi-Sq. values and *P*-values for linear mixed effects models specifying body site as a predictor variable, hyena identity as a random effect and an alpha-diversity metric as a dependent variable. The data are shown separately for adults and juveniles. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

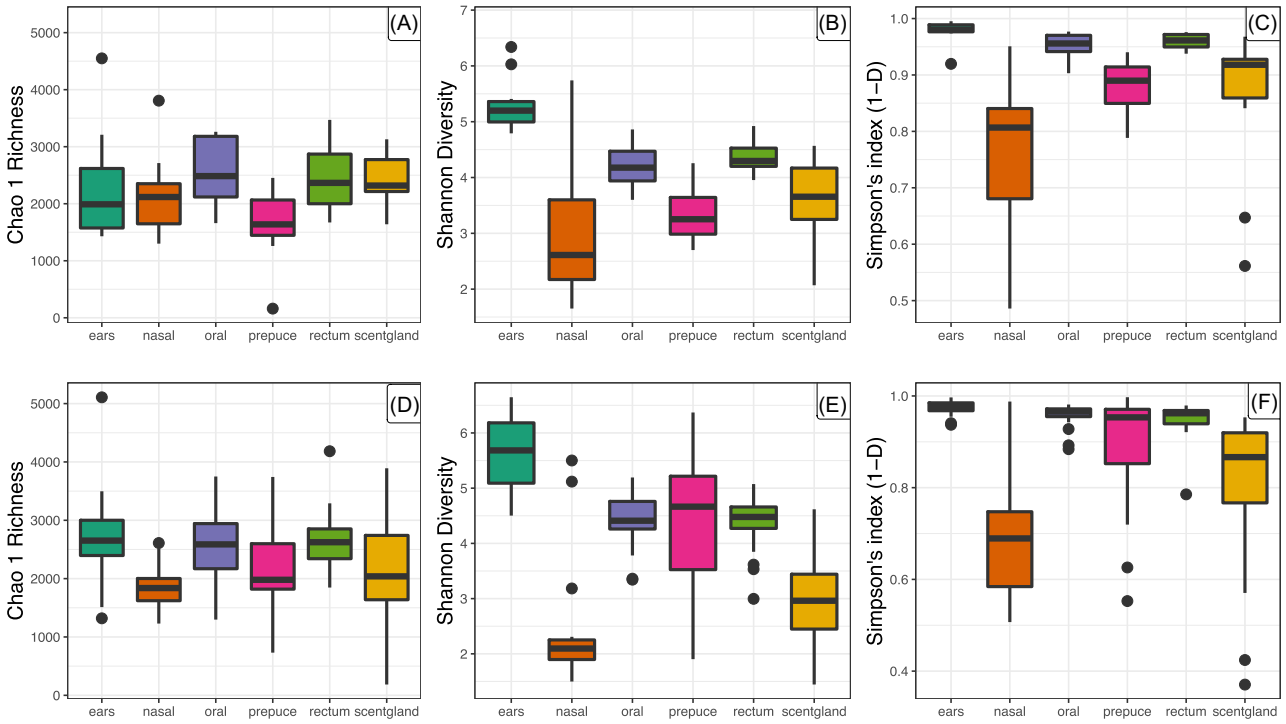

Figure 2. Body sites vary in their microbiota α-diversity. Box plots of microbiota alpha-diversity (Chao 1 richness, Shannon diversity, Simpson's index) at each body site in adults **(A–C)** and juveniles **(D–F)**. Boxed Xs are outlier values.

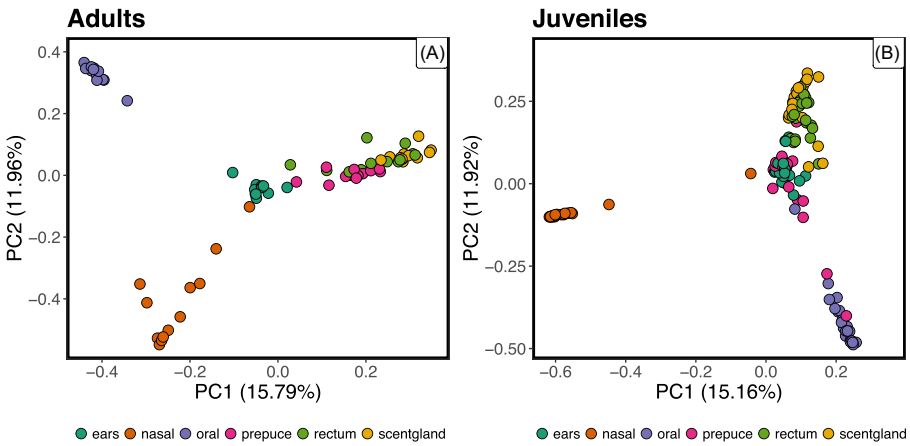

Figure 3. Microbiota cluster by body site in spotted hyenas. PCoA plots from Bray–Curtis dissimilarity matrices in **(A)** adults and **(B)** juveniles. Each point represents a sample and is color-coded by body site. Closeness of points indicates high community similarity. The % of variance accounted for by each principal coordinate axis is shown in the axis labels.

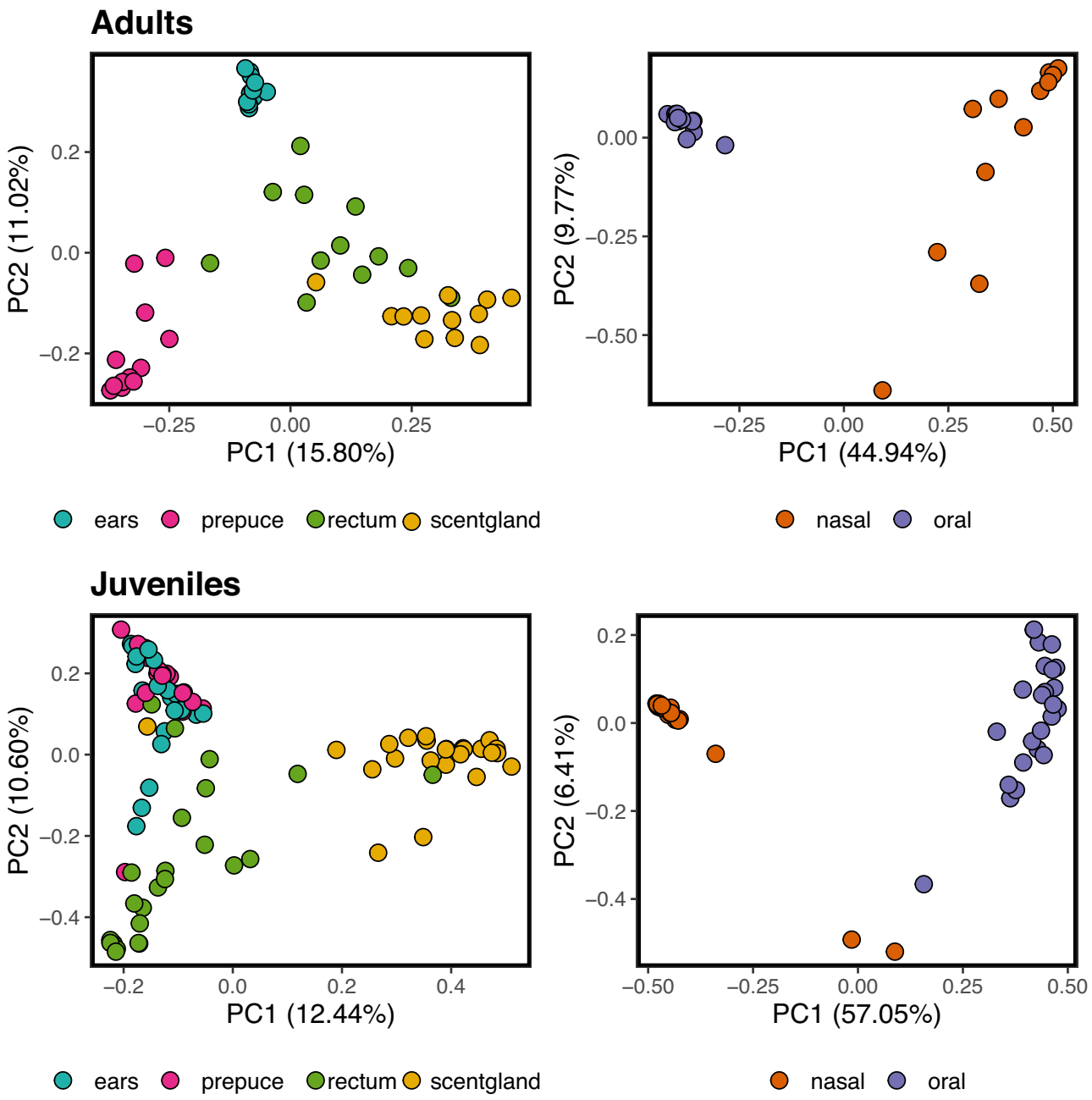

Figure 4. Microbiota structure and dispersion across body sites in juvenile and adult hyenas. PCoA plots from Bray–Curtis dissimilarity matrices in adults **(A, B)** and juveniles **(C, D)**. Because the nasal and oral microbiota were drastically different from those of other body sites (see Fig. 3), they are also plotted separately (B, D) in order to better visualize the variation in other body sites (A, C). Each point represents a sample and is color coded by body site. Closeness of points indicates high community similarity. The % of variance accounted for by each PCo axis is shown in the axis labels.

ordination shows that in adults, the microbiota from the ears, prepuce, anal scent gland and rectum formed unique, mostly non-overlapping clusters, but that in juveniles there was significant overlap between the ear and prepuce microbiota (Fig. 4).

Lastly, body sites also varied in their degree of microbial community dispersion in both adults and juveniles (PERMDISP Bray–Curtis, $F = 6.54$, $P < 0.0001$ for adults, $F = 27.81$, $P < 0.0001$ for juveniles; PERMDISP Jaccard, $F = 32.20$, $P < 0.0001$ for adults, $F = 38.26$, $P < 0.0001$ for juveniles). However, the differences were modest. In adults, the ear microbiota was more homogeneous among individuals than were microbiota at other body sites. In juveniles, the ear and prepuce microbiota showed less individual variation than the nasal and oral microbiota (Supplemental File 2: Table S9, Supporting Information).

Variation in microbiota profiles is significantly associated with host sex and age-class

Our results reveal that host sex and age-class were associated with variation in the microbiota of hyenas at multiple body sites. Among juveniles, microbiota richness differed between females and males across body sites (LMM Chao1 $\chi^2 = 4.79$, $P = 0.02$)

Table 2. Juvenile female hyenas have distinct anal scent gland microbiota compared to juvenile male hyenas.

Factor	Body site	R^2	*P*-value	Adjusted *P*-value
Bray–Curtis				
Sex	Ears	0.05	0.03	0.060
	Nasal	0.06	0.16	0.204
	Oral	0.038	0.17	0.204
	Prepuce	0.069	0.03	0.060
	Rectum	0.040	0.51	0.510
	Scent gland	**0.102**	**0.007**	**0.042**
Jaccard				
Sex	Ears	0.05	0.032	0.08
	Nasal	0.06	0.141	0.16
	Oral	0.042	0.48	0.48
	Prepuce	0.059	0.067	0.10
	Rectum	0.043	0.040	0.08
	Scent gland	0.08	0.011	0.06

Shown are the PERMANOVA tests assessing whether microbiota structure vary among the sexes (juvenile females vs juvenile males) in spotted hyenas. PERMANOVA tests based on Bray–Curtis (proportions of taxa) distance matrices are shown on top and those based on Jaccard (presence/absence) distance matrices are shown at the bottom. *P*-values were adjusted for multiple comparisons using the Benjamini–Hochberg method and set in bold if they were <0.05.

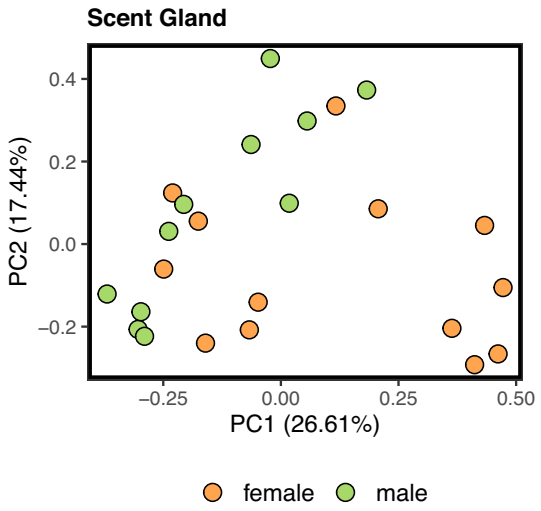

Figure 5. Juvenile females and juvenile males have distinct scent gland microbiota. PCoA plot from Bray–Curtis dissimilarity matrices showing the anal scent gland microbiota in juvenile females (orange) and juvenile males (green). Closeness of points indicates high community similarity. The % of variance accounted for by each PCo axis is shown in the axis labels.

but microbiota evenness did not (LMM Shannon $\chi^2 = 0.16$, $P = 0.68$; LMM Simpson $\chi^2 = 0.01$, $P = 0.91$). Specifically, juvenile males tended to have richer microbial communities than juvenile females. Additionally, host sex explained 10% of the structural variation in the anal scent gland microbiota (Table 2; Fig. 5). LEfSe indicated that juvenile males harbored greater abundances of unclassified *Clostridia*, *Prevotella* and unclassified *Firmicutes* in their anal scent glands, whereas juvenile females had more *Corynebacterium* and unclassified *Clostridiales* (Supplemental file 7: Table S10, Supporting Information).

The microbiota also differed between juvenile females and adult females (Bray–Curtis PERMANOVA $R^2 = 0.01$, $P = 0.001$; Jaccard PERMANOVA $R^2 = 0.008$, $P = 0.005$). However, the % variance in microbiota explained by age varied among body sites (Table 3). In the prepuce and rectum, host age-class accounted for 15 and 11% of the variation in microbiota structure, respectively (Table 3; Fig. 6). LEfSe analyses indicated that the prepuce microbiota of adult female hyenas, compared to those of juvenile females, were enriched in *Corynebacterium*, *Finegoldia* and *Clostridiales* (Supplemental file 7: Table S11, Supporting Information). In the rectum, adult females harbored greater abundances of *Anaerococcus* and *Corynebacterium*, whereas juveniles contained greater abundances of *Erysipelotrichaceae*, *Lachnospiraceae* and *Helicobacteraceae* (Supplemental file 7: Table S11, Supporting Information). Furthermore, the preputial microbiota of adult females but not those of other body sites tended to be more variable among individuals than did those of juvenile females (Bray–Curtis PERMDISP adults vs juveniles: ears $F = 0.66$, $P = 0.42$; nasal $F = 2.13$, $P = 0.15$; oral $F = 0.87$, $P = 0.35$; prepuce $F = 0.21$, $P = 0.02$; rectum $F = 0.006$, $P = 0.93$, anal scent gland $F = 1.67$, $P = 0.20$) (Supplemental file 2: Figure S4, Supporting Information). Lastly, no differences in alpha-diversity were evident between the microbiota of adult females and juvenile females across body sites (LMM Chao1 $\chi^2 = 0.15$, $P = 0.69$; Shannon $\chi^2 = 0$, $P = 0.99$; LMM Simpson $\chi^2 = 1.16$, $P = 0.28$).

Table 3. Adult female hyenas have distinct microbiota compared to juvenile female hyenas.

Factor	Body site	R^2	*P*-value	Adjusted *P*-value
Bray–Curtis				
Age-class	Ears	0.043	0.44	0.53
	Nasal	0.057	0.15	0.30
	Oral	0.033	0.72	0.72
	Prepuce	**0.151**	**0.001**	**0.006**
	Rectum	**0.113**	**0.004**	**0.012**
	Scent gland	0.04	0.37	0.53
Jaccard				
Age-class	Ears	0.045	0.111	0.166
	Nasal	0.042	0.26	0.26
	Oral	0.044	0.21	0.26
	Prepuce	**0.068**	**0.001**	**0.006**
	Rectum	**0.06**	**0.004**	**0.012**
	Scent gland	**0.048**	**0.021**	**0.042**

Shown are the PERMANOVA tests assessing whether microbiota structure vary among age-classes (adult females vs juvenile females) in spotted hyenas. PERMANOVA tests based on Bray–Curtis (proportions of taxa) distance matrices are shown on top and those based on Jaccard (presence/absence) distance matrices are shown at the bottom. *P*-values were adjusted for multiple comparisons using the Benjamini–Hochberg method and set in bold if they were <0.05.

Microbiota do not vary with host social rank, but are distinct among individuals

Neither microbiota α-diversity (LMM Chao1 $\chi^2 = 0.059$, $P = 0.80$; Shannon diversity $\chi^2 = 0.064$, $P = 0.80$; Simpson's index $\chi^2 = 0.16$, $P = 0.68$) nor β-diversity varied with host social rank in juvenile hyenas (both sexes included; Bray–Curtis Mantel test rho = 0.003, $P = 0.45$; Jaccard Mantel test rho = 0.028, $P = 0.19$). However, in both adult and juvenile hyenas, individual identity accounted for >11% of variation in microbiota structure across body sites (adult females: PERMANOVA $R^2 = 0.11$, $P = 0.001$ for Bray–Curtis, $R^2 = 0.15$, $P = 0.001$ for Jaccard; juvenile females and males: $R^2 = 0.12$, $P = 0.003$ for Bray–Curtis, $R^2 = 0.13$, $P = 0.01$ for Jaccard).

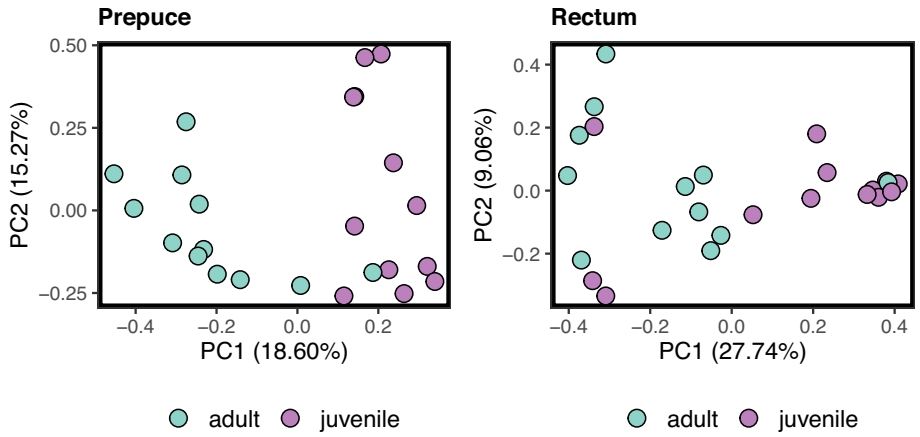

Figure 6. Adult females and juvenile females have distinct prepuce and rectal microbiota. PCoA plot from Bray–Curtis dissimilarity matrices showing the prepuce (left) and rectal (right) microbiota in adult females (turquoise) and juvenile females (purple). Closeness of points indicates high community similarity. The % of variance accounted for by each PCo axis is shown in the axis labels. Host age-class was significantly associated with PC1 in both the prepuce (LM $\beta = 0.43 \pm 0.05$, $P < 0.0001$) and rectum (LM $\beta = 0.30 \pm 0.10$, $P < 0.005$).

DISCUSSION

Principal findings of the study

The purpose of this study was to characterize the diversity and structure of microbiota at six distinct body sites in juvenile and adult spotted hyenas. We determined whether microbiota varied with host traits such as sex and social rank in juveniles of both sexes, and also whether microbiota differed between adult females and juvenile females. We found that the microbiota of spotted hyenas were body site specific, with respect to composition, structure and diversity in both adult females and juveniles. Despite the body site-specific structuring of the microbiota, these bacterial communities still exhibited strong specificity among individual hyenas, with host identity accounting for >11% of the total variation in microbiota structure. Additionally, the microbiota differed between adult females and juvenile females, particularly in the prepuce and rectum, indicating that age-related variation in diet, physiology and/or social interactions might underlie the differences in their microbial communities. Furthermore, the anal scent gland microbiota of juvenile females were distinct from those of juvenile males, suggesting the potential role of hormones or sex-specific life experiences in shaping these communities, even at this early life stage. Lastly, the microbiota of juvenile hyenas did not vary with host social rank. Future studies that include a larger number of adult hyenas of both sexes from a single clan will be required to determine whether social rank is associated with variation in the microbiota of mature hyenas.

Ecological theory and niche structuring of the microbiota in spotted hyenas

Our results support prior findings that anatomical body site predominantly structures microbiota α-diversity and β-diversity in mammals (e.g. Huttenhower *et al.* 2012; Carvalho *et al.* 2014; Alfano *et al.* 2015; Cheng *et al.* 2015; Bik *et al.* 2016; Dietrich *et al.* 2017; Chen *et al.* 2018). According to ecological theory, body sites often act as environmental filters and impede colonization and persistence of bacterial groups that do not possess suitable functional traits for surviving and competing in their respective environments (Costello *et al.* 2012; Nemergut *et al.* 2013; Widder *et al.* 2016; García-Bayona and Comstock 2018). Body sites are known to vary in their chemical and nutrient gradients, as well as in host immune activity (Huttenhower *et al.* 2012; Pereira and Berry 2017; Proctor and Relman 2017; Park 2018), and this likely contributed to the body site specificity of the microbiota observed in this study.

Why are the ear and oral microbiota highly diverse in spotted hyenas?

In terms of microbiota α-diversity, in both adults and juveniles, the ears and mouth were the most taxa rich, whereas the preputial and nasal communities were the least rich. In spotted hyenas, the oral and rectal microbial communities might be highly diverse due to hyenas' varied diet; hyenas eat various tissue types (e.g. skin, meat, bone, viscera) and prey species (Kruuk 1972; Hofer and East 1993; Cooper, Holekamp and Smale 1999), exposing them to many prey-associated microbiota and potentially giving rise to a diverse community of oral microbes that are able to utilize these varied substrates. A functional explanation for the high alpha-diversity of the hyena ear microbiota is lacking; however, one potential explanation is that hyena's ear is often immersed deep in carcasses. This may facilitate colonization by a wide diversity of bacteria. Indeed, the most abundant microbes from the hyena ear have been found in the skin and abdominal cavity of decomposing animals and the skin and hindgut of vultures (Roggenbuck *et al.* 2014; Cobaugh, Schaeffer and DeBruyn 2015; Metcalf *et al.* 2016). The nasal microbiota of hyenas might harbor low diversity due to the aerobic and mucous-rich environment of the nares, which may be inhospitable for many microbes (Biswas *et al.* 2015; Proctor and Relman 2017). Lastly, the hyena prepuce in both males and females is part of a long and narrow organ that has a small aperture; it is composed of thick epidermal tissue and likely contains sebaceous glands as in other carnivores (Kruuk 1972; Neaves, Griffin and Wilson 1980; Clapperton, Fordham and Sparksman 1987). This unique physiology and morphology might be contributing to the site's low bacterial diversity.

Microbiota composition of spotted hyenas compared to those of other mammals

With the exception of the ear, body sites harbored microbes that also reside in the oral cavity, skin, scent gland, genitalia and gut of other animals. A few dominant taxa of the hyena ear

microbiota (*Staphylococcaceae*, *Sphingobacteriaceae*) are generalist bacterial taxa that are present across skin sites in both terrestrial and aquatic mammals (Council *et al*. 2016; Chiarello *et al*. 2017; Ross *et al*. 2018; Ross, Rodrigues Hoffmann and Neufeld 2019). However, unlike the skin of humans and apes (Weese 2013; Council *et al*. 2016; Ross *et al*. 2018), the hyena's ear did not harbor appreciable numbers of *Corynebacterium* and *Propionibacterium*, and its microbiota profile only modestly resembled that of domestic dogs (Cuscó *et al*. 2017b; Ngo *et al*. 2018). Hyena noses and oral cavities exhibited fairly typical mammalian nasal and oral microbiota profiles (e.g. Mugisha *et al*. 2014; Alfano *et al*. 2015; Cheng *et al*. 2015; Adler *et al*. 2016; Dorn *et al*. 2017; Proctor and Relman 2017; Tress *et al*. 2017; Chen *et al*. 2018). The only difference was that hyena oral microbial communities did not contain significant numbers of *Prevotellaceae* and *Staphylococcaceae*, as these taxa are positively associated with diets high in carbohydrates, fruits and vegetables (Arumugam *et al*. 2011; Lory 2014; Sun *et al*. 2016; Li *et al*. 2017b), which hyenas eat very rarely, if at all.

In the prepuce, bacterial communities were dominated by taxa (i.e. *Corynebacterium*) that inhabit the urogenital and reproductive tract, skin and scent gland of other mammals (e.g. Ström Holst *et al*. 2003; Costello *et al*. 2009; Theis, Schmidt and Holekamp 2012; Courchay 2017; Leclaire *et al*. 2017; Li *et al*. 2018b; Ross *et al*. 2018). Additionally, the hyena rectum was inhabited by bacteria that typically reside in the guts of meat- and insect-eating mammals (Delsuc *et al*. 2014; Xue *et al*. 2015; Menke *et al*. 2017; Wasimuddin *et al*. 2017; He *et al*. 2018a; Han *et al*. 2019), as well as in dogs fed high-protein diets (Kim *et al*. 2017; Li *et al*. 2017b). Thus, although rectal microbiomes do not represent a proxy for gut microbiomes, it is not surprising to find that some taxa are shared between the two sites. Members of these bacterial families (i.e. *Lachnospiraceae*, *Streptococcaceae*, *Clostridiales_XI*) can ferment protein, and in the process, synthesize short-chain fatty acids (SCFAs), branched-chain amino acids, ammonia, phenols and indoles, which can then be used by the host and other bacteria (Dai, Wu and Zhu 2011; Yao, Muir and Gibson 2016; Korpela 2018; Diether and Willing 2019). Lastly, the anal scent gland microbiota profiles of hyenas strongly resembled the microbiota from other scent-producing areas in mammals (i.e. axillae, musk gland and scent glands; Theis, Schmidt and Holekamp 2012; Theis *et al*. 2013, 2016; Troccaz *et al*. 2015; Leclaire *et al*. 2017; Li *et al*. 2018b; Greene *et al*. 2019a). Dominant bacterial taxa in these regions (i.e. *Anaerococcus*, *Corynebacterium* and *Porphyromonas*) can also produce SCFAs (Albone and Shirley 1984; Ezaki *et al*. 2001; James, Hyliands and Johnston 2004; Sakamoto 2014); however, in scent-producing glands, SCFAs, as well as medium-chain fatty acids, are hypothesized to function as volatile odorants that are employed by their mammalian hosts during chemical signaling (Albone *et al*. 1974; Gorman, Nedwell and Smith 2009; Archie and Theis 2011; Carthey, Gillings and Blumstein 2018).

Microbiota of juvenile spotted hyenas vary with host sex but not social rank

Within the body sites of juvenile spotted hyenas, host sex was a significant predictor of microbiota composition and structure. Microbiota α-diversity differed between males and females across all of the surveyed body sites, and host sex also predicted microbial community structure in the anal scent gland. Similarly, sex differences have also been observed in the gut, skin and scent gland microbiota of primates, rodents, marsupials, carnivores, bats and marine mammals (e.g. Voigt, Caspers and Speck 2005; Theis *et al*. 2013; Leclaire, Nielsen and Drea 2014; Dominianni *et al*. 2015; Chiarello *et al*. 2017; Menke *et al*. 2017; Ren *et al*. 2017; Liu *et al*. 2018; Ross *et al*. 2018; Watson *et al*. 2019). In adult mammals, sex differences in the microbiota are often attributed to sex differences in physiology, morphology, hormones and behavior (Leclaire, Nielsen and Drea 2014; Dominianni *et al*. 2015; Grieneisen *et al*. 2017; Aivelo and Norberg 2018; Dietrich *et al*. 2018). In hyena societies, juvenile females associate with more individuals, and spend less time alone, than do juvenile males (Turner, Bills and Holekamp 2018). Male cubs are also known to scent mark more than female cubs, and male subadults scent overmark more than do subadult females (Theis *et al*. 2008). These early behavioral differences between the sexes might be modulating microbial exposure and consequently microbiota structure and composition in the anal scent gland of juvenile hyenas.

In juvenile hyenas, host social rank did not consistently predict microbiota profiles at any body site. Similarly, rank also failed to predict the gut microbiota in a Tanzanian population of spotted hyenas (Heitlinger *et al*. 2017). In contrast, the glandular microbiota of adult meerkats and sifakas have been found to vary with host social status (Leclaire, Nielsen and Drea 2014; Leclaire *et al*. 2017; Greene *et al*. 2019a). In our study, the 24 juvenile hyenas (11–21 months of age) were still in the process of developing their ranks; young hyenas typically do not assume their proper positions in the clan's hierarchy until they are at least 18 months of age (Holekamp and Smale 1993; Smale, Frank and Holekamp 1993). Future studies should investigate rank effects using a large sample of adult male and female hyenas from a single clan.

Differences between juvenile and adult microbiota profiles

Our results show that the microbiota of juvenile females and adult female hyenas differed in the prepuce and rectum, suggesting that life stage accounts for significant variation in the microbiota. This is also characteristic of primate, rodent, carnivore and marine mammal microbiomes (e.g. Sin *et al*. 2012; Smith *et al*. 2013; Langille *et al*. 2014; Leclaire, Nielsen and Drea 2014; Uchihashi *et al*. 2015; Theis *et al*. 2016; Chiarello *et al*. 2017; Jia *et al*. 2018; Mizukami *et al*. 2019; Reveles *et al*. 2019). Most notably, in the rectum, the microbiota of juvenile female hyenas were enriched in *Erysipelotrichaceae* and *Helicobacter*. High abundances of *Erysipelotrichaceae* have been associated with high-fat diets (Turnbaugh *et al*. 2008; Kaakoush 2015; Bermingham *et al*. 2017). Hyena milk has one of the highest fat contents of milks produced by land mammals (Hofer and East 1996), and some of the juveniles sampled here were still nursing, suggesting that perhaps this relatively high-fat diet might be related to the higher concentration of *Erysipelotrichaceae* in their rectums.

Individual identity predicts microbiota profiles in hyenas and other mammals

Despite the large amount of variation in microbiota profiles accounted for by body site, sex and age-class, individual hyenas still consistently harbored unique microbial communities. We found that individual identity was significantly associated with variation in the microbiota across all sampled body sites in both adults and juveniles, and accounted for >11% of the variation. In many mammals, host identity is one of the primary predictors

of the skin or gut microbiota (e.g. Wos-Oxley *et al.* 2016; Chiarello *et al.* 2017; Cuscó *et al.* 2017a,b; Raulo *et al.* 2017; Antwis *et al.* 2018; Trosvik *et al.* 2018; Kolodny *et al.* 2019). Individual differences in immune function, early-life experiences, social interactions and stress responses have been documented extensively for a range of mammalian taxa, and all of these variables may act individually or in concert to structure mammalian microbiomes (Ren 2016; Grieneisen and Archie 2017; Suzuki 2017; Björk *et al.* 2019).

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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AUTHOR CONTRIBUTIONS

KRT, KEH and CAR designed the study, Mara Hyena Project research assistants and KEH collected the samples and CAR extracted the genomic DNA. CAR, ADW and KRT analyzed the data, and CAR, KRT and KEH wrote the manuscript. All authors approved the final version of the manuscript.

ETHICS APPROVAL

Our research and procedures, which are described in the IACUC approval no. PROTO201900126, were most recently approved on 16 April 2019 and comply with the ethical standards of Michigan State University and Kenya.

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