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Special Feature

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Subtle diurnal microbial rhythms in a large mammalian carnivore

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Abstract

Mounting evidence suggests that the cyclic interaction between host cells and the gut microbiota orchestrates metabolic and immunological homeostasis throughout the day. Yet, examples of gut microbial rhythms in natural populations are scarce, limiting our understanding of their downstream consequences for host health, particularly in mammals that demonstrate strong co-evolutionary links with their microbiota. Furthermore, disregarding diurnal microbial variation restricts our ability to account and control for them in future studies. Here, we re-analyzed gut microbiota data from a 23-year longitudinal field study of 12 wild adult female spotted hyenas (*Crocuta crocuta*) in the Masai Mara National Reserve in Kenya to examine whether time of day was correlated with variation in gut microbial composition in this crepuscular–nocturnal carnivore. Overall, we found that gut microbial composition and structure, but not alpha diversity, slightly changed over the course of the day. Differences in microbiota composition between morning and afternoon became particularly apparent when restricting the analysis to the core microbiota (i.e., bacterial genera present in more than 85% of samples). Among the core microbiota, 11 genera—composed largely of the bacterial class Clostridia—varied in abundance with time of day, making this the second study to document gut microbial rhythms in a longitudinally sampled wildlife population. In contrast with the diurnal gut microbial oscillations of wild meerkats, those of hyenas are subtle, yet both species exhibit shifts specifically in the bacterial class Clostridia. This pattern implies that diurnal fluctuations are likely a characteristic of specific, common host-associated bacteria and their amplitude may be a product of host ecology. While our study detected diurnal trends, we encourage studies to employ a temporally denser sampling scheme. In this way, one can overlay short-term oscillations of the microbiome with information on host ecology and clarify consequences for the circadian phenotype of the host.

Key words: circadian rhythms, *Crocuta crocuta*, diurnality, gut microbiota, longitudinal study, microbial ecology, spotted hyenas, wildlife microbiome.

Cyclic and predictable environmental conditions entrain biological rhythms from days to seasons to years and even decades (Yerushalmi and Green 2009; Frick et al. 2018; Shuert et al. 2022). Abiotic and biotic changes within a 24-h period are among the most severe in nature (e.g., dark–light cycle, temperature oscillation, prey/predator activity). In response, mammals evolved self-sustaining circadian metabolic, immunological, and behavioral rhythms believed to be governed predominantly via "clock" genes (Asher and Schibler 2011; Man et al. 2016; Hazlerigg and Tyler 2019; Xiao et al. 2021). Aside from the genes in their own genomes, mammals—more so than other animals (Groussin et al. 2017; Mallott and Amato 2021)—rely on support from the myriad genes encoded by microbial symbionts (Moeller et al. 2016; Zepeda Mendoza et al. 2018; Cabral et al. 2022). It is now widely appreciated that many microbes, particularly those forming the hyperdiverse gut microbial community, are integral to the health of their mammalian host (Sommer et al. 2017) and influence their metabolism, e.g., Kishino et al. (2013); immunity, e.g., Brooks et al. (2021); behavior, e.g., Ezenwa et al. (2012); and ecology, e.g., Song et al. (2020), resulting in a constant and reciprocal cross-talk between host and bacterial cells (Frazier and Chang 2020). Such cross-talk is easiest when the rhythmic proliferation of bacteria is synchronized to the circadian rhythm of the host (Schmid et al. 2023).

Only few reports exist that describe diurnal rhythms in the gut microbial community and its interaction with host-mediated circadian rhythms in mammals. We know, however, from work on humans and captive mice that genetics (i.e., clock genes) together with feeding and light cues synchronize cyclic host-directed and microbiome-mediated processes to maintain metabolic and immunological homeostasis throughout the day (Thaiss et al. 2014, 2016;

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Table 1. Sample sizes for all 12 hyenas split by morning and afternoon.

HyenaID	No. of samples	Years represented by samples	Mean samples per year	No. of samples during morning	No. of samples during afternoon
M1	13	1993 to 1999	1.9 (±1.1 SD)	8	5
D1	34	1999 to 2001, 2003 to 2007, 2011	3.8 (±2.6 SD)	9	25
G1	33	2003 to 2010, 2012 to 2015	2.9 (±1.7 SD)	8	25
M2	33	1997 to 2007, 2009 to 2012	2.2 (±2.4 SD)	14	19
D2	24	2006 to 2007, 2009 to 2014, 2016	2.7 (±0.5 SD)	7	17
G2	17	2011 to 2016	2.8 (±1.7 SD)	5	12
M3	14	1993 to 1995	4.7 (±5.1 SD)	6	8
D3	48	1995 to 2009, 2011 to 2012, 2015	2.7 (±2.0 SD)	32	16
G3	16	2011, 2013 to 2016	3.2 (±2.0 SD)	6	10
M4	18	1993 to 1995, 1997 to 2000	2.6 (±1.8 SD)	9	9
D4	27	1994 to 1997, 2000 to 2004, 2006	2.7 (±1.3 SD)	14	13
G4	23	2006, 2008 to 2014	2.9 (±2.4 SD)	4	19

Kaczmarek et al. 2017; Teichman et al. 2020; Tognini et al. 2020; Tuganbaev et al. 2020; Brooks et al. 2021), and that mice lacking gut microbiota show dampened circadian rhythmicity in metabolism and immunity (Leone et al. 2015). Feeding is thought to be particularly important in prompting gut microbiota to engage with host cells (Thaiss et al. 2014; Tuganbaev et al. 2020; Schmid et al. 2023). In wild meerkats, 13 out of the 16 core bacterial genera fluctuated, reaching an up to 10-fold difference in absolute abundances between samples collected in the morning, shortly after foraging begins, and those gathered in the evening (Risely et al. 2021). The common bacterial phylum Bacillota (formerly known as Firmicutes), and especially members of its bacterial class Clostridia, are frequently found to fluctuate (Leone et al. 2015; Thaiss et al. 2016; Shaani et al. 2018; Risely et al. 2021). Overall, between 10% and 40% of resident gut microbes are estimated to oscillate throughout the day (Thaiss et al. 2014; Zarrinpar et al. 2014; Reitmeier et al. 2020). Yet, whether gut microbial rhythms are common across mammalian hosts with diverse ecologies is unclear.

Spotted hyenas (*Crocuta crocuta*) are long-lived, crepuscular–nocturnal carnivores that reside throughout sub-Saharan Africa. They live in hierarchical, matrilineal social groups containing females, their cubs, and immigrated breeding males (Frank 1986; Holekamp et al. 2012). The gut microbial community of both captive and wild hyenas was studied previously using 16S rRNA gene sequencing (Heitlinger et al. 2017; Chen et al. 2020; Rojas et al. 2020, 2023) and shotgun metagenomics (Rojas et al. 2023). In the latter study, the gut microbiota of 12 wild adult females belonging to 4 matrilines from the Masai Mara National Reserve was characterized from 13 to 49 fecal samples per individual that were opportunistically collected between 1993 and 2016 (Rojas et al. 2023). The researchers identified several bacterial core genera that were found in >85% of the fecal samples. Among these core taxa were many genera belonging to the bacterial class Clostridia, but also Bacteroidia and Actinobacteria. The study concluded that hyenas have strongly individualized gut microbial communities, but that these communities also show variation associated with host age and, to some degree, matriline (Rojas et al. 2023).

Hyenas also exhibit strong diurnality with 95% of their activity falling between 5 PM and 9 AM (Kolowski et al. 2007; Cozzi et al. 2012). The diurnality in activity of these carnivores raises the possibility of rhythmicity in gut microbial dynamics, even though their feeding times are sporadic (Holekamp et al. 1997). This is unlike meerkats for whom foraging activity peaks early in the morning and again shortly before sunset, resulting in strong gut microbial rhythms (Risely et al. 2021). Yet, we still might expect subtle differences between the gut microbial community of hyenas at the onset of their active period in the late afternoon, representing a fastened gut microbial community, compared with the gut microbial community in the morning, representing a more metabolically active microbiota. For that reason, we re-analyzed the previously published longitudinal microbiota data from wild hyenas residing in the Masai Mara National Reserve in southwestern Kenya (Rojas et al. 2023) to determine whether gut microbial diurnal dynamics are detectable in this large carnivore. The presence and strength of gut microbial rhythms in this carnivore will advance our understanding of the role of host-associated bacteria in supporting the circadian phenotype of the host.

Materials and methods.

Data collection.

Previously published hyena microbiome data came from 12 adult females from a single social group that were sampled between 1993 and 2016 (Rojas et al. 2023). In brief, all longitudinal samples were collected in the Masai Mara National Reserve in southwestern Kenya. The Mara–Serengeti ecosystem experiences 2 dry seasons (late December to March and late June to mid-November) and 2 rainy seasons (late November to early December and April to early June; Green et al. 2019). Hyena fission–fusion dynamics and large home ranges meant sampling was opportunistic and yielded uneven sample sizes per individual (mean 25.1 ± 10.5 SD; Table 1). Individual hyenas were sampled for 2 to a maximum of 22 years of their life (Rojas et al. 2023). Hyenas were identified based on their unique spot patterns, allowing assignment of fecal samples to individual hyenas and their metadata on matriline, social rank, and age at sample collection (Holekamp et al. 1999; Rojas et al. 2023). Importantly, sample collection occurred during the morning and afternoon, and the precise time of day (24 h) was recorded. A total of 292 samples fell within the active period of 5:00 PM to 9:00 AM: 176 samples were recovered between 5:19 PM and 8:00 PM, whereas 115 were gathered between 5:33 AM and 8:54 AM (Table 1). Another 7 samples were collected between 9:04 and 10:09 AM, and 2 in the early afternoon; all of which were included in our analyses. One sample with an unknown sampling time was excluded from all analysis. In the field, all fecal samples were stored in cryogenic vials in liquid nitrogen before transport to Michigan State University on

dry ice, where they were kept at −80 °C (Rojas et al. 2023). In this study, we are assuming that fecal microbiotas are representative of intestinal microbiomes, as it was shown to be the case in freshly collected and properly stored samples for other mammal species (Menke et al. 2015, 2017).

16S rRNA sequencing and bioinformatic processing.

Previously published paired-end 16S rRNA gene sequences (V4 region; 250 bp) were processed in the same way as in the original publication (Rojas et al. 2023). Briefly, they were imported into RStudio (v3.6.2; R Core Team 2022) and quality filtered and assigned to amplicon sequence variants (ASVs) using the Divisive Amplicon Denoising Algorithm (DADA2 v1.14.1) package (Callahan et al. 2016). The SILVA rRNA gene reference database (v132; Quast et al. 2013) was then used to assign ASVs to their respective taxonomy down to the species level if possible (Johnson et al. 2019). If sequences were unable to be classified down to genus and species level, we used their last known classification (e.g., family). ASVs designated as Eukarya, chloroplasts, or mitochondria were manually removed, and the package decontam (v1.6.0) filtered out 4 bacterial ASVs that were more commonly found in the extraction blanks than in biological samples (Davis et al. 2018). All singletons were removed. Two samples containing fewer than 100 reads were not included in the final data set (final $n = 300$). The remaining samples averaged 13,088 reads (±5,178 SD) each.

Statistical analyses.

All statistics and visualizations were performed in R studio (v4.2.1). We first calculated the time since sunrise for each sample using the *getSunlightTimes*() function from the "suncalc" package (v0.5.2; Thieurmel and Elmarhraoui 2024). We then visualized the diversity of important gut bacterial classes and identified common core genera, as defined in Risely (2020), found in 85% of samples using the *core*() function from the "microbiome" package (v1.18; Lathi et al. 2017) on samples agglomerated to genus level. We calculated observed ASV richness and Shannon diversity using the function *estimate_richness*() from the "phyloseq" package (v1.42.0; McMurdie and Holmes 2013), and Faith's phylogenetic diversity employing the *pd*() function from the "picante" package (v1.8.2; Kembel et al. 2010) on data rarefied to 2,900 reads (McMurdie and Holmes 2014; Weiss et al. 2017; Schloss 2024). Compared with the number of observed ASVs, which is a measure of bacterial richness, Shannon diversity weighs ASVs by their abundance and thus estimates the richness and evenness of the bacterial community. Faith's phylogenetic diversity additionally considers taxonomic dissimilarity between ASVs and therefore resembles the phylogenetic breadth of the bacterial community. The phylogenetic tree of bacterial ASVs necessary to compute Faith's phylogenetic diversity was constructed with DECIPHER (v2.14.0; Wright 2016) and phangorn (v2.5.5; Schliep 2011). We explored temporal dynamics in alpha diversity with generalized additive models (GAMs) using the *gam*() function of the "mgcv" package (v1.8.41; Wood 2017) since we predict temporal variation to be nonlinear (Risely et al. 2021). The model contained sample collection time as a smooth (e.g., hours after sunrise) or categorical (e.g., morning vs. afternoon) explanatory variable, age as a smooth explanatory variable, and matriline as a factor. To account for interindividual differences, hyena identity was included as a random factor. Model diagnostics were assessed using the *gam.check*() function of the "mgcv" package.

To compare beta diversity across samples collected at different times of day, we first calculated unweighted and weighted Unifrac distances using the *distance*() function from the "phyloseq" package on rarefied data (Cameron et al. 2021). Both distances consider phylogenetic distance between ASVs, but weighted Unifrac scales for evenness based on ASV abundance, whereas unweighted Unifrac is computed on an ASV presence–absence matrix and, hence, places more weight on rare taxa. In other words, weighted Unifrac represents the structure of the bacterial community, while unweighted Unifrac is a measure of its composition. Additionally, we calculated both distances using only data from core genera since we predict that bacterial genera commonly found across host individuals are more likely to be impacted by host circadian rhythms. We tested for statistical differences in beta diversity with PERMANOVAs using the *adonis2*() function from the "vegan" package (v2.6-4; Oksanen et al. 2022). The models contained the explanatory variables hours after sunrise, age, and matriline—the latter 2 factors shown to be relevant in a previous publication (Rojas et al. 2023). In these models, interindividual differences were accounted for by specifying hyena identity as a strata term. These models were also run using Bray–Curtis and Aitchison distances; results were unchanged and thus are not shown. We tested for any potential influences of heterogeneity of group variances (Anderson and Walsh 2013) using the *betadisper*() function in the "vegan" package. The data were visualized with the *plot_ordination*() function in the "phyloseq" package.

We also assessed the impact of hours since sunrise, age, and matriline while controlling for hyena identity and sequencing depth on the abundances of individual bacteria taxa using a generalized linear latent variable model (GLLVM) from the "gllvm" package (Niku et al. 2019). In comparison with traditional abundance-based analyses (e.g., ANCOM), joint species distribution models such as GLLVMs account for correlations between bacterial taxa when predicting their abundance with respect to the explanatory variable. GLLVMs also allow more complex model structures. Importantly, this model was computed on unrarefied but centered log-ratio (CLR) normalized read count data as proxy for the abundance of certain bacterial genera (Quinn et al. 2019). Another set of GAMs (Wood 2017) assessed linear and nonlinear changes on the (CLR-transformed) abundances of the genera identified by the GLLVMs. Due to the spread of the data clustering around the morning and late afternoon, we repeated the analyses using time of day as a categorical variable (i.e., morning/afternoon) instead of the continuous "hours since sunrise" variable, but it did not change the results.

Results

Gut microbial composition, alpha-, and beta diversity.

We sought to examine diurnal oscillations in the gut microbiota of 12 hyenas opportunistically sampled over a period of 23 years (Table 1). A total of 300 fecal samples were included in our composition, alpha- and beta-diversity analyses. Bacteria of the class Clostridia (53.5%) were most common, and together with Bacilli (15.1%), Actinobacteria (7.8%), Bacteroidia (7.4%), Fusobacteria (5.1%), and Erysipelotrichia (4.9%) made up more than 90% of all recovered reads from hyena fecal samples (Fig. 1A). A total of 26 genera comprised the common core (found in >85% of samples) in the gut microbial community of hyenas, of which the majority are members of the class Clostridia (Fig. 1B). Neither sample collection time—measured as hours since sunrise, nor time of day—categorized as morning or afternoon—affected the gut microbiota alpha diversity calculated from rarefied data (collection time: GAMs $P > 0.05$; Fig 2A; Supplementary Data SD1). Observed ASVs and Shannon varied between matrilines in line with previous reports (GAMs $P < 0.05$; Supplementary Data SD1).

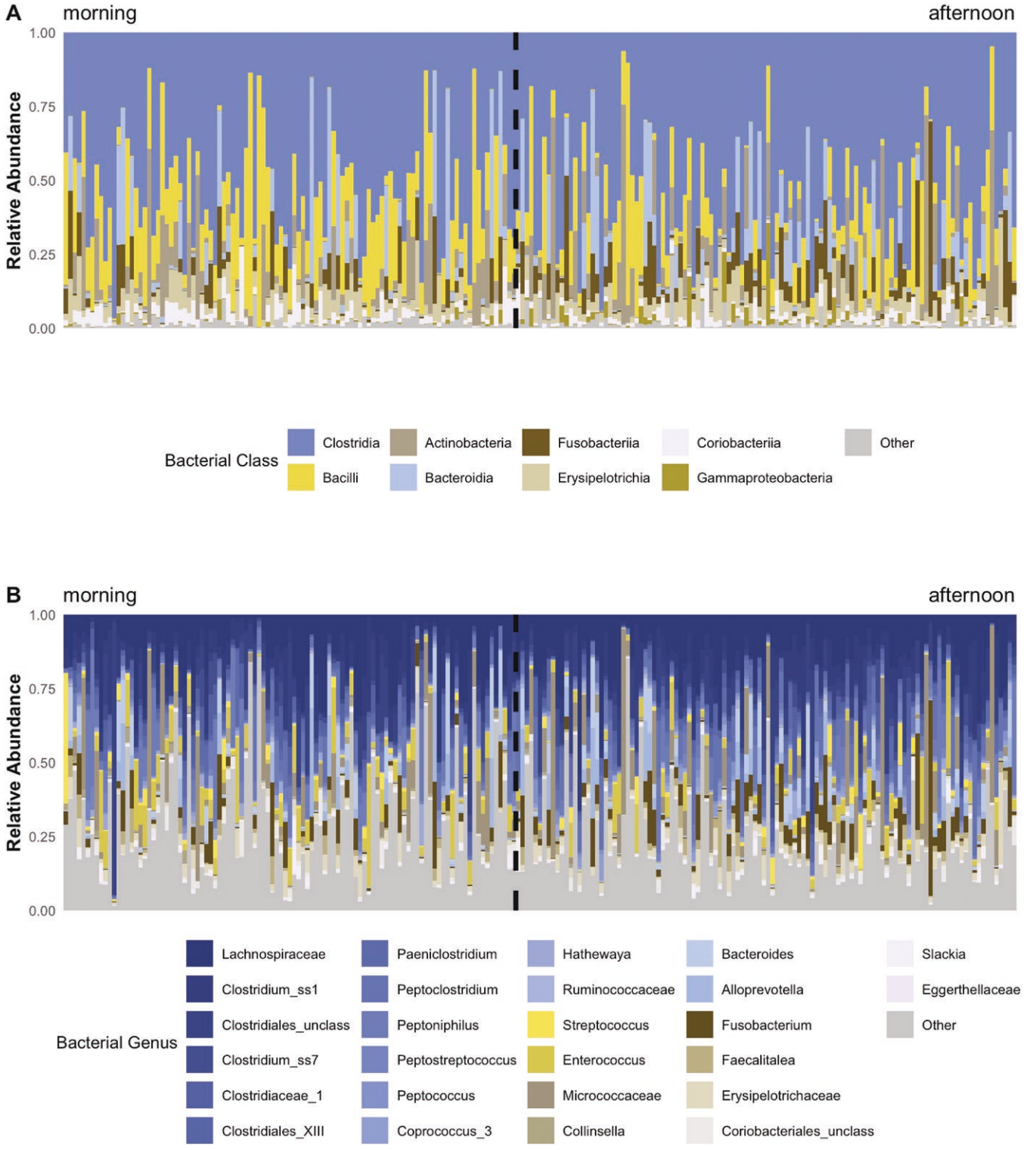

Fig. 1. Compositional differences in A) bacterial classes and B) bacterial core genera between all samples ordered by hours since sunrise. Dashed line separates samples taken in the morning from those collected in the afternoon. Colors in the core genera plot reflect to which bacterial class the genera belong (e.g., *Streptococcus* is a hue derived from the color associated with its bacterial class). unclass—unclassified; ss1—sensu stricto 1; ss7—sensu stricto 7.

Hours since sunrise (and daytime) emerged as a significant contributor in shaping gut microbial community composition (measured as unweighted Unifrac distances; PERMANOVA with continuous collection time, $F = 3.44$, $R^2 = 0.011$, $P = 0.001$; Supplementary Data SD2) and, to a lesser extent, structure (measured as weighted Unifrac distances; PERMANOVA with continuous collection time, $F = 3.01$, $R^2 = 0.010$, $P = 0.065$; Supplementary Data SD2). When restricting analysis to the 26 core genera, gut microbial community composition (Fig. 2B; PERMANOVA with continuous collection time, $F = 4.32$, $R^2 = 0.014$, $P = 0.007$) and structure (PERMANOVA with continuous collection time, $F = 2.97$, $R^2 = 0.010$, $P = 0.046$) were both significantly influenced by the hours after sunrise (and daytime; Supplementary

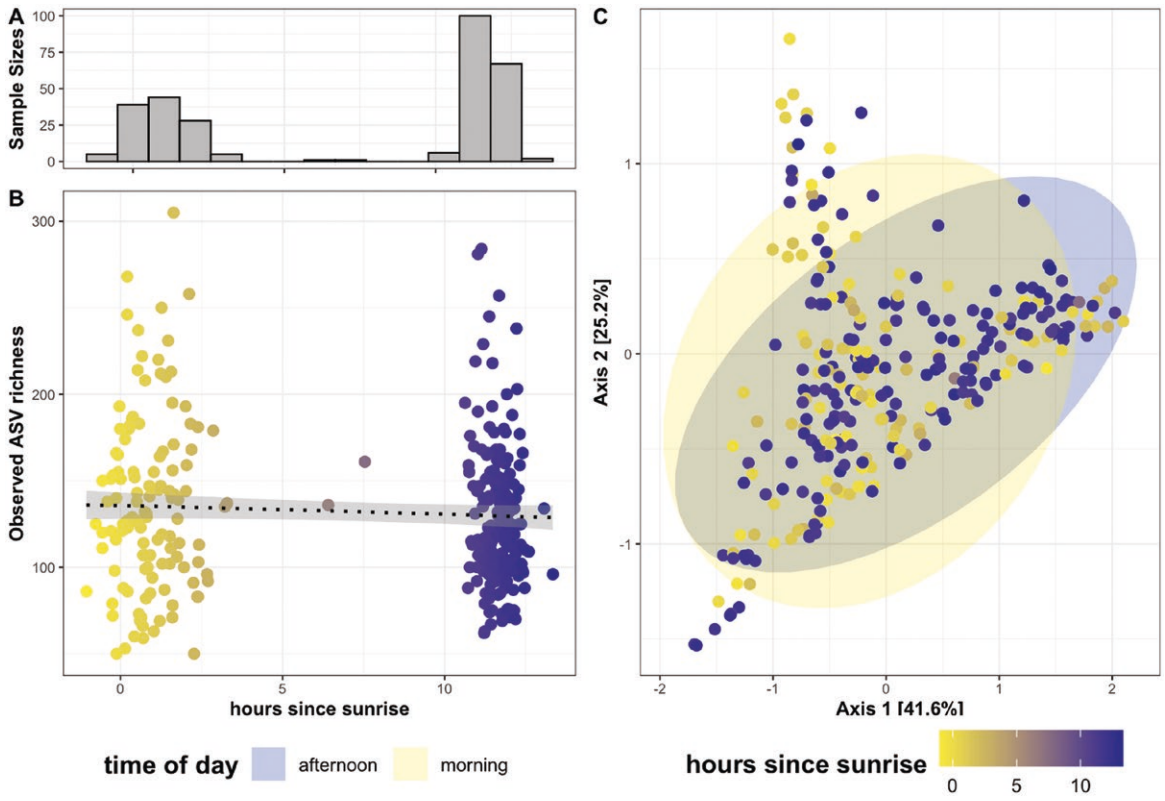

Fig. 2. A) Sample distribution throughout the day. B) Gut microbiota alpha diversity (shown as observed ASVs) does not fluctuate with the number of hours since sunrise, whereas C) community composition (shown here as unweighted Unifrac calculated from core genera) and structure (not shown here) differ between samples collected in the morning and those collected in the afternoon.

Data SD2). Differences in heterogeneity of group dispersion were not observed for core microbial community composition and structure (core unweighted Unifrac—collection time, $F = 0.74$, $P = 0.955$; core unweighted Unifrac—time of day, $F = 2.56$, $P = 0.111$; core weighted Unifrac—collection time, $F = 1.27$, $P = 0.105$; core weighted Unifrac—time of day, $F = 0.09$, $P = 0.771$), but when analyzing the entire microbial community (rarefied unweighted Unifrac—collection time, $F = 9.59$, $P < 0.001$; rarefied unweighted Unifrac—time of day, $F = 4.67$, $P = 0.031$; rarefied weighted Unifrac—collection time, $F = 2.65$, $P < 0.001$; rarefied weighted Unifrac—time of day, $F = 0.20$, $P = 0.656$).

Temporal variation in the abundance of core genera.

The GLLVM identified 11 bacterial genera that varied in abundance between morning and afternoon (Fig. 3A). *Streptococcus*, *Enterococcus*, and *Collinsella* were, for example, more abundant shortly after sunrise, whereas *Faecalitalea*, *Peptococcus*, and *Peptoclostridium* were more abundant in the afternoon. Some core genera, such as *Clostridium* sensu stricto 1 or *Slackia*, decreased with hyena age, and several genera were more common in specific matrilines (Supplementary Data SD3).

When testing for linear or nonlinear changes in bacterial abundances using GAMs (Fig. 3B), *Streptococcus* (edf = 6.3, $F = 2.05$, $P = 0.040$) and *Enterococcus* (edf = 1.00, $F = 15.43$, $P < 0.001$) declined in abundance with hours after sunrise. Another 6 genera increased in abundance in the afternoon: *Faecalitalea* (edf = 1.61, $F = 4.61$, $P = 0.013$), *Ruminococcaceae* (edf = 1.0, $F = 7.20$, $P = 0.008$), *Peptococcus* (edf = 1.0, $F = 13.65$, $P < 0.001$), *Coprococcus_3* (edf = 1.0, $F = 19.31$, $P < 0.001$), *Clostridiales_XIII* (edf = 1.0, $F = 7.02$, $P = 0.009$), and *Peptoclostridium* (edf = 1.7, $F = 6.44$, $P = 0.002$). Most notably, the majority of core genera were members of the bacterial class Clostridia (phylum Bacillota). Differences between matrilines were rare. All 11 genera varied with age in a nonlinear manner (Supplementary Data SD4).

Discussion

Diurnal microbial rhythms have been relatively unexplored in gut microbiome research, which is surprising given that their rhythmic interaction with host cells likely supports key metabolic and immunological functions of the host (Frazier and Chang 2020; Schmid et al. 2023). Here we report subtle but consistent diurnal variation in gut microbial composition and structure of a wild crepuscular–nocturnal carnivore, the Spotted Hyena. Specifically, the microbial composition, structure, and abundances of several core bacterial genera changed from the morning, which marks the end of the active period, to the afternoon, when hyenas start foraging again. These findings represent only the second instance of gut microbial rhythms detected in wildlife. Neglecting the role of diurnal variation in wildlife microbiome research likely underestimates the importance of host-associated microbiota (Allaband et al. 2024).

A total of 11 core genera primarily belonging to the bacterial class Clostridia differed in abundance between samples taken in the morning and afternoon, suggesting that diurnal oscillations are characteristic of specific hyena-associated gut bacteria and are not exhibited by all gut bacteria. Bacillota, and especially Clostridia, are common in hyena guts (Heitlinger et al. 2017; Chen et al. 2020; Rojas et al. 2023) and therefore likely fulfill important functional roles in these carnivores including nutrient sequestration (Levin et al. 2021; Zoelzer et al. 2021; Cabral et al. 2022), immunomodulation (Ferreira et al. 2021; Berman et al. 2023), and exclusion of potential pathogens (Spragge et al. 2023). However, in comparison with the only other example of gut microbial rhythms, found in wild

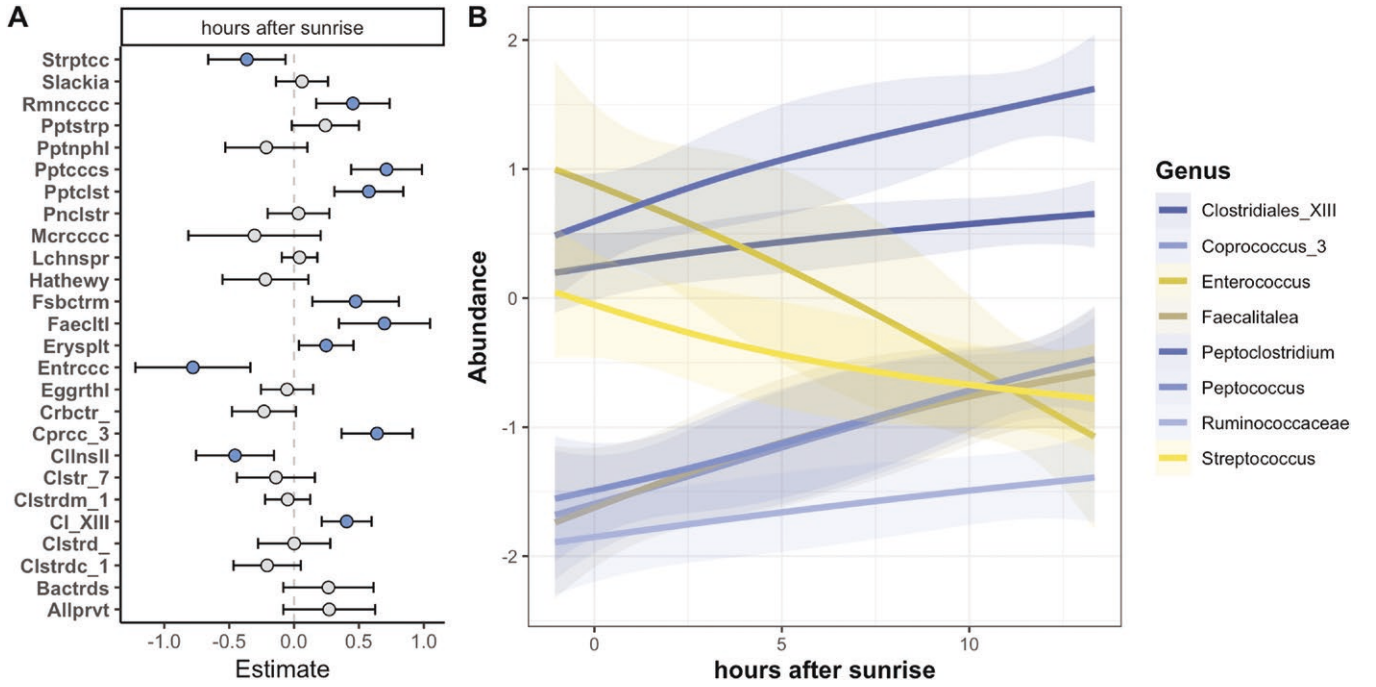

Fig. 3. A) Plot of GLLVM beta estimates for each bacterial taxon tested and B) CLR-transformed abundances of candidate core genera plotted against hours after sunrise. Statistically significant differences are marked by a blue dot, and only significant linear effects were depicted. Colors are the same in the compositional bar plot and reflect to which bacterial class the genera belong (e.g., *Streptococcus* is a hue derived from the color associated with its bacterial class). Streptcc = *Streptococcus*, Slackia = *Slackia*, Rmncccc = *Ruminococcaceae*, Pptstrp = *Peptostreptococcus*, Pptnphl = *Peptoniphilus*, Pptcccs = *Peptococcus*, Pptclst = *Peptoclostridium*, Pnclstr = *Paeniclostridium*, Mcrcccc = *Micrococcaceae*, Lchnspr = *Lachnospiraceae*, Hathewy = *Hathewaya*, Fsbctrm = *Fusobacterium*, Faecltl = *Faecalitalea*, Erysplt = *Erysipelotrichaceae*, Entrccc = *Enterococcus*, Eggrthl = *Eggerthellaceae*, Crbctr_= *Coriobacteriales*_unclass, Cprcc_3 = *Coprococcus*_3, Cllnsll = *Collinsella*, Clstr_7 = *Clostridium*_ss7, Clstrdm_1 = *Clostridium*_ss1, Cl_XIII = *Clostridiales*_XIII, Clstrd_= *Clostridiales*_unclass, Clstrdc_1 = *Clostridiaceae*_1, Bactrds = *Bacteroides*, Allprvt = *Alloprevotella*.

meerkats (Risely et al. 2021), the diurnal variation in gut microbial composition of hyenas is subtle, and only accounted for up to 1.5% of the variation compared with 5% to 15% in meerkats. In meerkats, diurnal differences greatly outweigh many other long-term trends including host age (Risely et al. 2021), individual differences (Risely et al. 2022), and selection by the environment (Risely et al. 2023). Yet, in meerkats too, the genera showing the most extreme diurnal fluctuations were members of the bacterial class Clostridia (Risely et al. 2021). Moreover, the genus *Peptococcus* became more abundant at the onset of the active period in both meerkats and hyenas. In experimental studies, Bacillota are frequent oscillators (Thaiss et al. 2014; Reitmeier et al. 2020), implying that diurnal fluctuations are likely characteristic of certain tightly host-associated bacteria.

Actinomycetota (formerly known as Actinobacteria) are another bacterial phylum common in the gut microbiotas of hyenas (Heitlinger et al. 2017; Chen et al. 2020; Rojas et al. 2023). The bacterial genus *Collinsella*, within the Actinomycetota, showed a lower abundance in the afternoon. Actinomycetota are likely sensitive to feeding times (Shaani et al. 2018) and *Collinsella* was found to be favored in a carnivorous diet low in fiber (Gomez-Arango et al. 2018). Feeding times and diet undoubtedly have consequences for mammalian gut microbial composition (Kartzinel et al. 2019) and rhythmicity (Brooks et al. 2021). However, the time of feeding is opportunistic in hyenas with only about a third of all hunting attempts resulting in kills (Holekamp et al. 1997; Cooper et al. 1999), which means that a fecal sample collected in the morning may at times still reflect a fastened rather than a fed microbial community. This pattern is unlike meerkats, which feed daily (Risely et al. 2021). We suspect that regular feeding times explain why the diurnal gut microbial rhythms of meerkats are so much more pronounced than in hyenas. An alternative explanation for the less pronounced gut microbial rhythms in hyenas could be the sparse sample coverage. Gathering feces from the same hyena is especially difficult due to their fission–fusion dynamics and large home ranges (Smith et al. 2008; Rojas et al. 2023). However, sample sizes as low as 4 per individual sufficed to detect strong diurnal fluctuations in meerkats (Risely et al. 2021). Taken together, our findings illustrate apparent but subtle diurnal rhythms in hyenas, which we think is explained by the sporadic foraging times of these large carnivores.

Because of their tight co-evolutionary history, one might even hypothesize that gut microbial rhythms albeit of varying strengths could be a universal feature of the symbiotic relationship between mammals and their gut bacteria (Groussin et al. 2017; Song et al. 2020; Mallott and Amato 2021; Schmid et al. 2023; Worsley et al. 2024). What would the presence of gut microbial rhythms mean for future studies exploring the gut microbiota of mammals? For one, if unaccounted for, gut microbial rhythms may introduce methodological bias. A recent study found that ignoring diurnal variation in gut microbial composition affected the conclusions drawn from each experiment that the authors re-analyzed (Allaband et al. 2024). In other words, diurnal variation in the gut microbiota must be accounted for in order to ascertain which factors truly shape the gut microbial community beyond the effects of daily fluctuations. One possibility is to limit sampling to specific time windows in the day, and heavily sample all individuals during this time. This strategy would however require comprehensive knowledge about the circadian ecology and behavior of hosts, e.g., feeding bouts and gut transit times (Asnicar et al. 2021). Moreover, in field studies such as ours, this design is often neither practical nor feasible. Another option is to account for sampling times statistically such as is done for other methodological variation, e.g., storage and batch effects (Menke et al. 2015; Gibbons et al. 2018). This strategy might be particularly realistic in the move toward more model-based statistical analyses (Fountain-Jones et al. 2024)

such as joint species distribution models, which allow complex model structures.

Alternatively, daily microbial rhythms could inform about recurring physiological, immunological, and ecological challenges faced by mammals and which bacterial groups perform important services to their mammalian host throughout the day. They could represent a plastic and adaptive response similar to those found in hibernating mammals, which experience seasonal changes in the gut microbial community reflecting their immediate metabolic needs (Dill-McFarland et al. 2014; Sommer et al. 2016). So, rather than treating microbial diurnal oscillations as noise, many interesting questions can be asked about gut microbial rhythms in relation to the biology and ecology of its mammalian host (Schmid et al. 2023), e.g., to what extend do gut microbial rhythms support circadian metabolism and immunity; what factors disturb microbial rhythms and what are the consequences for host fitness; how might seasonal changes interact with diurnal microbial rhythms to impact host health; and how much does host ecology influence microbial rhythmicity? With regard to the latter, our work suggests that the irregular foraging behavior of large carnivores such as hyenas could limit the rhythmicity of their gut microbiota relative to animals for which feeding times are more predictable.

Supplementary data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1. Generalized additive model results for 3 metrics of alpha diversity.

Supplementary Data SD2. PERMANOVA results comparing inter-sample dissimilarity.

Supplementary Data SD3. Generalized linear latent variable model results for core genera.

Supplementary Data SD4. Generalized additive model results for 11 candidate genera.

Supplementary Data SD5. Raw metadata table.

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Author contributions

Conceptualization, investigation, methodology, validation, writing—original draft: DWM and CAR. Data curation: CAR. Formal analysis, visualization: DWM, CAR, and AR. Funding acquisition: DWM, CAR, and KRT. Project administration: DWM; Supervision: AR and KRT. Writing—review & editing: all authors.

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Conflict of interest

None declared.

Data availability

Raw 16S rRNA gene sequence files are accessible from the NCBI's Sequence Read Archive, under BioProject PRJNA733503 and accession numbers SAMN19468262 to SAMN19468578. The code and an RDS data file concerning the analysis of circadian rhythms can be accessed from https://github.com/DominikWSchmid/CircadianRhythms_SpottedHyenas. All bioinformatics pipelines and scripts from the original publication can be found at https://github.com/rojascon/HyenaGutMicrobiome_AcrossGenerations. The sample metadata is also accessible as Supplementary Data SD5.

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