Check for updates

Received: 8 September 2023 | Revised: 12 April 2024 | Accepted: 17 April 2024

DOI: 10.1111/mec.17370

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Determinants of microbiome composition: Insights from free-ranging hybrid zebras (*Equus quagga* × *grevyi*)

Joel O. Abraham[1] | Bing Lin[1,2] | Audrey E. Miller[1] | Lucas P. Henry[1,3] | Margaret Y. Demmel[1,4] | Rosemary Warungu[5] | Margaret Mwangi[5] | Patrick M. Lobura[5] | Luisa F. Pallares[1,6,7] | Julien F. Ayroles[1,6] | Robert M. Pringle[1] | Daniel I. Rubenstein[1,5]

[1]Department of Ecology and Evolutionary Biology, Princeton University, Princeton, New Jersey, USA

[2]School of Public and International Affairs, Princeton University, Princeton, New Jersey, USA

[3]Department of Biology, New York University, New York City, New York, USA

[4]Section of Ecology, Behavior and Evolution, University of California San Diego, San Diego, California, USA

[5]Mpala Research Conservancy, Laikipia County, Kenya

[6]Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey, USA

[7]Friedrich Miescher Laboratory, Max Planck Society, Tübingen, Germany

Correspondence
Joel O. Abraham and Daniel I. Rubenstein, Department of Ecology and Evolutionary Biology, Princeton University, Princeton, NJ, USA.
Email: joeloa@princeton.edu and dir@princeton.edu

Funding information
High Meadows Environmental Institute, Princeton University; National Science Foundation, Grant/Award Number: 2019256075 and DEB-2225088

Handling Editor: Kayla King

Abstract

The composition of mammalian gut microbiomes is highly conserved within species, yet the mechanisms by which microbiome composition is transmitted and maintained within lineages of wild animals remain unclear. Mutually compatible hypotheses exist, including that microbiome fidelity results from inherited dietary habits, shared environmental exposure, morphophysiological filtering and/or maternal effects. Interspecific hybrids are a promising system in which to interrogate the determinants of microbiome composition because hybrids can decouple traits and processes that are otherwise co-inherited in their parent species. We used a population of free-living hybrid zebras (*Equus quagga* × *grevyi*) in Kenya to evaluate the roles of these four mechanisms in regulating microbiome composition. We analysed faecal DNA for both the *trn*L-P6 and the 16S rRNA V4 region to characterize the diets and microbiomes of the hybrid zebra and of their parent species, plains zebra (*E. quagga*) and Grevy's zebra (*E. grevyi*). We found that both diet and microbiome composition clustered by species, and that hybrid diets and microbiomes were largely nested within those of the maternal species, plains zebra. Hybrid microbiomes were less variable than those of either parent species where they co-occurred. Diet and microbiome composition were strongly correlated, although the strength of this correlation varied between species. These patterns are most consistent with the maternal-effects hypothesis, somewhat consistent with the diet hypothesis, and largely inconsistent with the environmental-sourcing and morphophysiological-filtering hypotheses. Maternal transmittance likely operates in conjunction with inherited feeding habits to conserve microbiome composition within species.

KEYWORDS
diet, environmental DNA (eDNA) metabarcoding, Grevy's zebra (*Equus grevyi*), hybrid, maternal effects, microbiome, plains zebra (*Equus quagga*)

Bing Lin and Audrey E. Miller contributed equally and are listed alphabetically.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2024 The Authors. *Molecular Ecology* published by John Wiley & Sons Ltd.

1 | INTRODUCTION

Gut microbiomes are important for organismal function in a diverse suite of animals (D'Argenio, 2018; Neish, 2009; Sommer & Bäckhed, 2013). Among other roles, symbiotic microbes in the digestive tract break down food and toxins and convert them into forms that their hosts can assimilate (Bäckhed et al., 2005; Neish, 2009; Schluter & Foster, 2012). The gut microbiome is of particular importance for herbivorous mammals, which rely on bacteria to extract energy and nutrients from their cellulose-rich food, synthesize vitamins and amino acids that they cannot obtain from their diets, and detoxify plant defensive compounds (Dearing & Kohl, 2017; Hammer et al., 2019; Kohl et al., 2014; Muegge et al., 2011; Neish, 2009). Indeed, mammalian herbivores derive nearly all of their nutrition from the microbial communities in their gut (Dearing & Kohl, 2017; Muegge et al., 2011) and die without their microbial symbionts (Kohl et al., 2014). Studies of mammalian herbivore microbiomes consistently find that microbiome composition is highly conserved within species and that closely related species have more similar microbiomes (Brooks et al., 2016; Groussin et al., 2017; Ley et al., 2008; Nishida & Ochman, 2018), even in diverse assemblages of sympatric herbivores that intermingle under natural conditions (Kartzinel et al., 2019). However, because organisms lack a gut microbiome during embryonic development (Blaser & Dominguez-Bello, 2016; Koenig et al., 2011; Tanaka & Nakayama, 2017), they must acquire the microbes that constitute their microbiome during and after birth (Bäckhed et al., 2005; Bergström et al., 2014; D'Argenio, 2018; Koenig et al., 2011; Rosshart et al., 2019; Tanaka & Nakayama, 2017), a process that remains poorly understood (Baniel et al., 2022; Blaser & Dominguez-Bello, 2016; Brooks et al., 2016; Groussin et al., 2017; Ley et al., 2008).

There are several complementary hypotheses for how microbiome fidelity might be maintained within species. First, because some aspects of diet are phylogenetically conserved, microbiome composition might be preserved within species by inherited dietary habits (H_1) (Abraham et al., 2022; Codron et al., 2019; Kartzinel et al., 2019; Muegge et al., 2011; Pansu et al., 2022). Microbiome composition has been shown to covary with diet in both captive and free-ranging mammals (David et al., 2014; Kartzinel et al., 2019; Muegge et al., 2011; Nielsen et al., 2023; Sanders et al., 2015); animals may source their microbiome directly from the foods they eat, or different diets may select for distinct microbial communities that can metabolize those foods and/or tolerate the gut conditions necessary for their digestion (Hammer et al., 2019; Kohl et al., 2014; Schluter & Foster, 2012; Zhang et al., 2016). Second, microbiome composition might be shaped by species-specific interactions with the environment (H_2): even closely related species exhibit distinctive habitat-use and movement patterns in nature (Abraham et al., 2022; Daskin et al., 2023; Elith & Leathwick, 2009; Noonan et al., 2020) and might thus be exposed to unique suites of microbes due to microbial turnover across space (Grieneisen et al., 2019; Metcalf et al., 2017; Tasnim et al., 2017; Yatsunenko et al., 2012). Although the species-specificity of microbiome composition among captive animals suggests that H_2 alone is not a sufficient explanation, it may play a contributing role in wild populations, which have not been as intensively studied. Third, species' morphological and/or physiological traits might act as a filter on the composition of their gut microbiomes (H_3) (Amato et al., 2019; Godon et al., 2016; Sommer & Bäckhed, 2013; Song et al., 2020). Differences in gut size, temperature and chemistry, for example, might constrain the microbial community in the gut (Amato et al., 2019; Godon et al., 2016; Sommer & Bäckhed, 2013; Song et al., 2020). Last, microbiome composition might be determined via maternal effects (H_4) (Bäckhed et al., 2005; D'Argenio, 2018), through early exposure to the mother's microbiome during parturition, nursing and other maternal care (Baniel et al., 2022; Bergström et al., 2014; Blaser & Dominguez-Bello, 2016; Koenig et al., 2011; Tanaka & Nakayama, 2017). Evaluating the relative support for these hypotheses is difficult, because they are not mutually exclusive and are all linked within a given species (Brooks et al., 2016; Groussin et al., 2017; Yatsunenko et al., 2012).

While controlled manipulative experiments are ideal for mechanistic inference, they are impractical for large, wild ungulates. Hybrid animals—the product of mating between different species—are emerging as valuable systems for understanding the species-specificity of microbiomes in a wide range of taxa, including ungulates (Grieneisen et al., 2019; Li et al., 2016; Miller et al., 2021; Nielsen et al., 2023). Among other things, the study of hybrid microbiomes offers inroads for exploring the impacts of genes, behaviour and environment on the microbiome in natural ecological contexts, because hybrids decouple traits and processes that are otherwise co-inherited or coupled. Hybrids are often phenotypically intermediate and more phenotypically variable than their parent species, and are thus distinct from either parent (Harrison, 1990; Russell, 1941). Also, natural hybrids can only occur where both parent species are present (Harrison, 1990; Russell, 1941), thereby reducing potentially confounding effects of environmental variation (Metcalf et al., 2017; Tasnim et al., 2017). Wild hybrids can therefore be viewed as natural pseudo-experiments for testing influences of diet, environment, morphophysiology and maternal effects on microbiome composition. Yet naturally occurring hybrids are rare, and there have been correspondingly few prior studies on their gut microbiota (but see Grieneisen et al., 2019; Li et al., 2016; Miller et al., 2021; Nielsen et al., 2023).

We studied a naturally occurring population of hybrid zebras in Laikipia, Kenya (Cordingley et al., 2009). These hybrid zebra are always the products of mating between male Grevy's zebra (*Equus grevyi*, a globally endangered species) and female plains zebra (*Equus quagga*, a near-threatened species) (Cordingley et al., 2009; Schieltz & Rubenstein, 2015). Hybrid offspring are morphologically intermediate between the two species (Figure 1a) and exhibit behaviours distinct from either parent species (Schieltz & Rubenstein, 2015). All zebras are monogastric hindgut fermenters; thus, in contrast to ruminant herbivores, the site of fermentation is near the end of the digestive tract, such that the faecal microbiome is generally representative of the microbial community responsible for digestion and fermentation (Costa & Weese, 2012; Metcalf et al., 2017; Reed

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

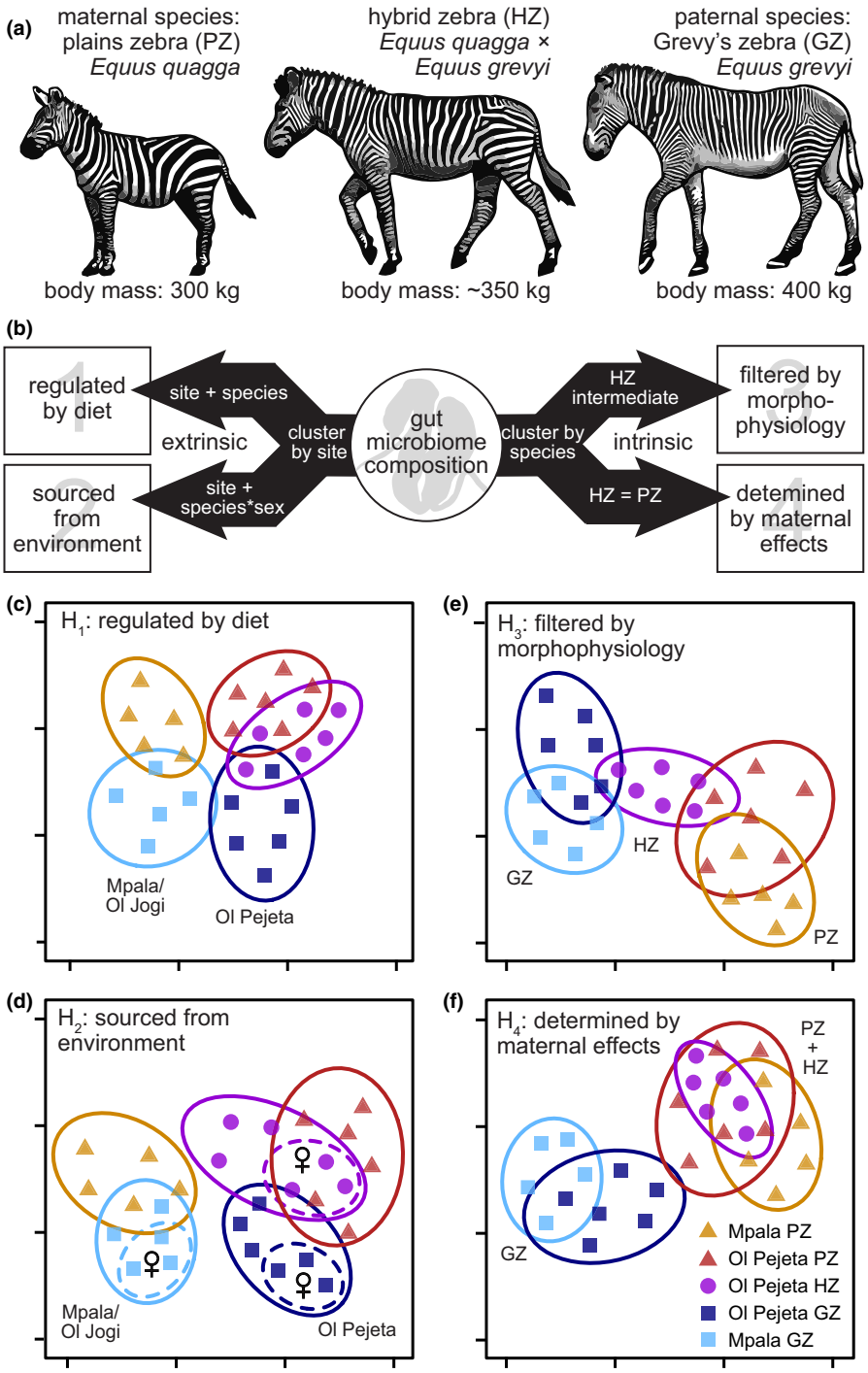

FIGURE 1 Hypothesized determinants of microbiome composition in hybrid zebra. (a) Hybrid zebra (HZ) are always the products of mating between male Grevy's zebra (GZ) and female plains zebra (PZ) and are morphologically intermediate between the two species. (b) Conservation of microbiome composition within species might arise via multiple mechanisms; although these mechanisms are not mutually exclusive, each of them suggests a distinct set of relationships between the microbiome composition of HZ and Ol Pejeta or Mpala PZ and GZ (schematized here as hypothetical ordinations where proximity of points reflects degree of similarity). (c) If diet proximately regulates microbiome composition, then patterns of microbiome composition should parallel patterns of diet composition, which should differ across locations and between species. (d) If environmental attributes regulate microbiome composition, then location should be a primary axis of microbiome differentiation, as should sex for GZ and HZ due to their sex-specific landscape use. (e) If host morphophysiology filters microbiome composition, then HZ microbiomes should be compositionally intermediate to their parent species, because HZ are morphologically intermediate. (f) If maternal effects regulate microbiome composition, then HZ microbiomes should resemble those of PZ, as HZ are always born to PZ mothers. Panels (c–f) are mock ordinations representing expected patterns of microbiome composition under the different hypotheses.

et al., 2017; Stothart et al., 2023). These attributes, coupled with a relatively large body of knowledge on equids and their microbiomes (Costa & Weese, 2012; McKinney et al., 2020; Metcalf et al., 2017; Reed et al., 2017; Rubenstein, 1986; Stothart et al., 2023), make hybrid zebras a useful system in which to probe the determinants of microbiome composition.

Building on the general hypotheses outlined above, we tested a series of specific predictions about how the microbiomes of zebra hybrids might relate to those of their parent species (Figure 1b). Under a strong influence of diet (H_1), patterns of microbiome composition should parallel patterns of diet composition (Kartzinel et al., 2019), which in turn should differ across locations and between species (Kartzinel et al., 2015; Pansu et al., 2022) (Figure 1c). Under a strong environmental influence (H_2), sampling location should be a principal determinant of microbiome composition (Couch et al., 2020), and sex should have a significant effect on the microbiomes of hybrid and Grevy's zebra due to their sex-specific habitat use (Cordingley et al., 2009; Rubenstein, 1986) (Figure 1d). If microbiome composition is determined by host morphophysiology (e.g. body size and hence gut volume, metabolic rate, etc.; Duque-Correa et al., 2021; Godon et al., 2016) (H_3), then the hybrid microbiome should be compositionally intermediate between the two parent species and

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

should also be more variable than those of their parent species (Figure 1e). Finally, if maternal effects determine microbiome composition (H_4), then hybrid microbiomes should be less variable than those of their parent species, should be nested entirely within those of the maternal species (always plains zebra) (Nielsen et al., 2023), and microbiome composition should cluster by host species overall, irrespective of location (Figure 1f). Thus, despite the infeasibility of controlled experimentation in these imperilled large herbivores, the contrasting patterns predicted by four priorly defined hypotheses (Figure 1) provide a basis for evaluating the influences of different but mutually compatible mechanisms of microbiome maintenance.

2 | MATERIALS AND METHODS

2.1 | Study system

In January 2020, we collected faecal samples from plains, Grevy's and hybrid zebras in two localities in Laikipia County, central Kenya (Figure 2). Laikipia encompasses ~10,000 km^2 of conservancies, communal rangelands and private ranches (Georgiadis et al., 2007). Mean annual rainfall across the region ranges from ca. 500–900 mm, increasing with elevation from north to south (Georgiadis et al., 2007). Our sampling localities were 20 km apart along this rainfall gradient and therefore differ in mean annual rainfall by >250 mm (Figure 2). To the north, two neighbouring conservancies, Mpala and Ol Jogi, jointly encompasses ca. 440 km^2 (Kartzinel et al., 2019), with mean annual rainfall of ~640 mm in the area where sampling was conducted (Alston et al., 2022). Ol Pejeta spans ~360 km^2 to the south and receives ~900 mm mean annual rainfall (Georgiadis et al., 2007). The two sampling localities further differ in soil, consequently supporting distinct vegetation communities. Within the Mpala/Ol Jogi complex (henceforth, 'Mpala'), sampling was conducted on red sandy alfisols, which supports a mosaic of diverse, structurally variable thorn-scrub savanna dominated by three *Acacia* (sensu lato) species with a patchy understory (Goheen et al., 2013). Ol Pejeta occupies exclusively clay-rich vertisols and is more open, with patches of *Acacia s.l.* and *Euclea* savanna interdigitating large open grasslands (Fischhoff et al., 2007). Dominant grasses across both locations include *Cynodon* spp., *Themeda triandra* and *Pennisetum* spp. (Fischhoff et al., 2007).

2.2 | Study species

Grevy's and plains zebra occur sympatrically in grasslands and savannas of north-central Kenya (Cordingley et al., 2009; Rubenstein, 1986). However, plains zebra are more water-dependent (Kihwele et al., 2020) and consequently favour cooler and wetter environments than do Grevy's zebra (Cordingley et al., 2009; Rubenstein, 1986). Grevy's zebra (400 kg) are also ~30% larger than plains zebra (300 kg) (Soria et al., 2021) (Figure 1a). Hybrids are smaller than Grevy's zebra but larger than plains zebra and exhibit other intermediate traits (Schieltz & Rubenstein, 2015) (Figure 1a). Plains and Grevy's zebra exhibit markedly different social structures (Schieltz & Rubenstein, 2015). Plains zebra form herds composed of multiple harems (each with a stallion, several females and dependent offspring) as well as groups of bachelor males (Cordingley et al., 2009; Rubenstein, 1986). As a result, male and female space use is similar for plains zebras. In contrast, landscape use is sex-specific

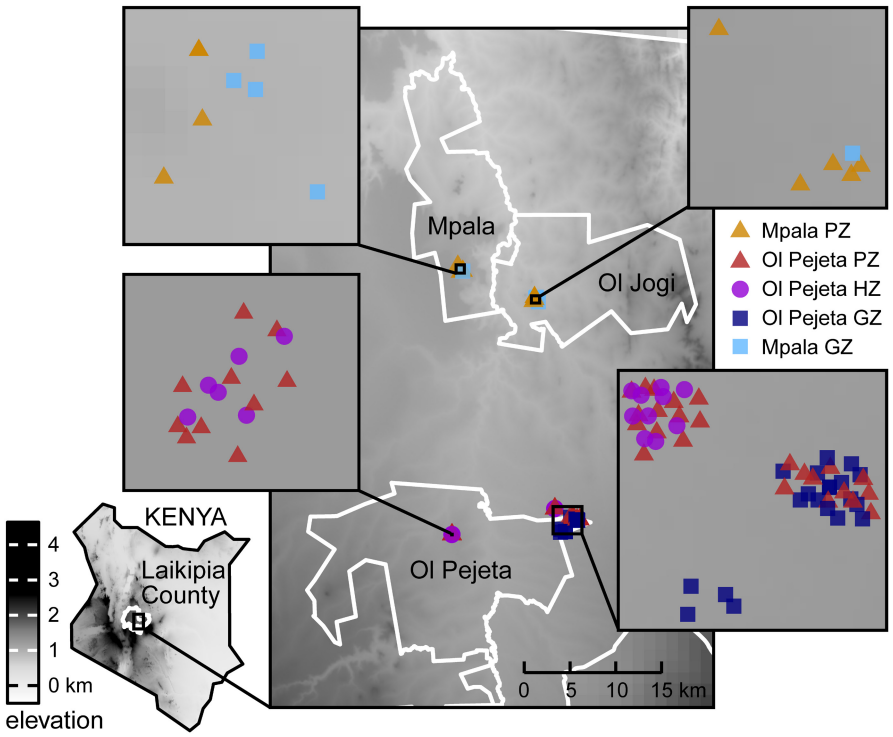

FIGURE 2 Map of study locations and sample collection locations. Faecal sampling was conducted at two locations: the adjacent Mpala and Ol Jogi conservancies to the north and Ol Pejeta Conservancy 20 km to the south. The background is coloured by elevation, which increases from north to south, with a corresponding gradient of increasing mean annual rainfall across the study region. Point colours correspond to the five zebra subpopulations, point shapes correspond to the three zebra species.

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

for Grevy's zebra, with territorial males and more nomadic females: male Grevy's zebra control access to unstable groups of females by defending areas with key resources, such as waterholes and high-quality forage patches (Cordingley et al., 2009; Rubenstein, 1986). Because they are born exclusively to plains zebra mothers, hybrids are raised entirely in plains zebra society (Cordingley et al., 2009). Hybrid females continue to behave like plains females into adulthood, whereas hybrid males sometimes establish territories as Grevy's males do (Schieltz & Rubenstein, 2015), such that hybrid space use is also somewhat sex-specific. Although Grevy's and plains are both strict grazers, their diets differ significantly in the relative abundance of different grass taxa (Kartzinel et al., 2015; Pansu et al., 2022), possibly as a consequence of their morphological and behavioural differences. The diets of hybrid zebra have not previously been characterized.

2.3 | Sample collection

All hybrid zebra samples were collected from Ol Pejeta, the only place where these hybrids are known to occur, while plains and Grevy's samples were collected from both sampling localities (Figure 2). At Ol Pejeta, Grevy's zebras are kept in a separate fenced-off section of the reserve to protect them from predators and prevent further introgression with hybrids (Schieltz & Rubenstein, 2015). Hybrid samples were collected from the main reserve, all within 10 km of where Grevy's zebra samples were collected, and plains samples were collected from both areas of Ol Pejeta (Figure 2).

Hybrid zebra and their natal harems were opportunistically located each day, informed by scouting reports and previous days' sampling and ranging patterns. All hybrid zebras and their affiliated harems were known prior to sampling (Cordingley et al., 2009; Schieltz & Rubenstein, 2015). We tried to sample each known hybrid once and to collect a faecal sample from both a male and female adult from the same harem; for each hybrid faecal sample collected, at least one sample from an adult plains zebra of the same sex as the hybrid was collected on the same day.

Once located, zebras were followed at a distance until they defecated, after which the faecal sample was collected immediately. We only collected faecal material from the center of each dung pile (i.e. faecal material not directly in contact with the ground or any surrounding vegetation). Samples were placed in sterile containers with 70% ethanol and stored in a cooler for transport to the lab. We took photographs (right flank) of each zebra individual from which samples were obtained to avoid pseudo-replication from repeated sampling of the same individual. We also recorded GPS locations and metadata including time, location, species, sex, age (adult vs. juvenile), group structure, habitat/vegetation type, and individual behaviour.

Altogether, we collected 88 samples from five 'subpopulations' (Table S1), defined by the location where samples were collected: Ol Pejeta hybrids ($n = 16$), Ol Pejeta plains ($n = 34$), Ol Pejeta Grevy's ($n = 22$), Mpala plains ($n = 9$) and Mpala Grevy's ($n = 7$).

2.4 | DNA sequencing and bioinformatics

We used laboratory procedures and bioinformatics pipelines employed in previous faecal metabarcoding studies of large herbivores in central Kenya and elsewhere (Kartzinel et al., 2015, 2019; Pansu et al., 2022). Samples were stored at −20°C prior to DNA extraction. DNA was extracted in a clean laboratory at Mpala Research Centre using Zymo Research Quick-DNA Faecal/Soil Microbe kits according to manufacturer's instructions. Samples were processed in small batches (4–27; typically 6) with one extraction control (sample-free extract) per batch to check for contamination. We conducted PCR on all samples in triplicate, to enable quality assessment via comparison of technical replicates, as well as on all extraction and PCR controls (nuclease-free water instead of DNA extract). For diet analysis, we amplified the P6 loop of the chloroplast *trn*L(UAA) region, using indexed primers *g* and *h* (Kartzinel et al., 2015, 2019; Pansu et al., 2022; Taberlet et al., 2007). For microbiome analysis, we amplified the V4 hypervariable region of the 16S rRNA gene with indexed Illumina primers 515F/806R (Kartzinel et al., 2019). Because each sample and control was uniquely barcoded during library preparation, diet and microbiome PCR products (and their respective negative controls) were pooled in separate libraries for purification. Diet and microbiome libraries were purified with MinElute™ purification kits (Qiagen, MD, USA). Purified libraries were then sequenced on an Illumina MiSeq (2 × 200 bp paired-end reads) at the Genomics Core Facility in Princeton University's Lewis-Sigler Institute for Integrative Genomics. We obtained 3,901,345 reads from the diet sample library and 6,081,642 reads from the microbiome sample library.

Diet sequence data were curated using the OBITools v2 package (Boyer et al., 2016), while microbiome sequence data were processed using the DADA2 v1.18 big data pipeline (Callahan et al., 2016), implemented in R v4.0.2 (R Core Team, 2020). In both cases, we assembled paired-end reads, assigned sequences to their original samples, merged identical sequences, and then removed low-quality sequences and those likely to have resulted from PCR or sequencing errors (sequences <8 bp and those with nucleotide ambiguities or mean Illumina fastq quality scores <30) as well as sequences represented only once in the entire data set. We identified and eliminated chimeras (sequences potentially resulting from PCR and/or sequencing errors). For diet data, we identified chimeras using the *obiclean* command (with parameters $d = 1$ and $r = 0.25$) in OBITools, which determines whether a sequence is more likely to be a true sequence ('head'), a sequence derived from another one ('internal'), or a sequence from which no other sequence is derived and is not derived from another ('singleton'). We discarded all sequences that were more frequently categorized as derivative ('internal') than 'head' or 'singleton' ($n = 3835$ sequences). For microbiome data, we identified and removed chimeras using the *removeBimeraDenovo* command in DADA2 ($n = 2054$ sequences). For both diet and microbiome data, we identified putative contaminants by comparing the abundance of sequences between samples and negative controls; we discarded any sequences that were more abundant in negative controls than in samples ($n = 41$ and 88 sequences for diet and microbiome data).

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Outlying PCR replicates were identified and discarded by comparing dissimilarity distributions between vs. within replicates (see Pansu et al., 2022). We discarded any samples with only one PCR replicate remaining after filtering. For the remaining samples, we averaged the number of reads per sequence across PCR replicates.

We then assigned taxonomic identifications to sequences. For diet analysis, all unique sequences retained after filtering were designated as molecular operational taxonomic units (mOTUs) and taxonomic assignments were made by comparison to a reference library containing 460 of the roughly 500 plant species known to occur at Mpala (Gill et al., 2019) as well as a global reference library generated from the European Molecular Biology Laboratory database (Ficetola et al., 2010). We eliminated sequences ($n = 579$) that did not perfectly match a sequence in either reference library (which we consider justified given the near-comprehensive coverage of the local library) to further eliminate chimeras, PCR artefacts, and sequencing errors (Kartzinel et al., 2019). For microbiome analysis, amplicon sequence variants (ASVs) were assigned to taxonomic classifications using the SILVA SSU v138.1 bacterial reference database (Yilmaz et al., 2014). ASVs derived from chloroplasts or mitochondria were removed, as were ASVs identified as non-bacterial or unidentified at the kingdom level ($n = 2297$ sequences). The sequences of remaining bacterial ASVs were then used to construct a phylogenetic tree using QIIME2 (Bolyen et al., 2019): bacterial sequences were aligned, the alignment was masked to reduce ambiguity, and the phylogeny was constructed with the command *fasttree*.

We then rarefied the number of reads in each sample to account for differences in sequencing depth, using the average across 1000 iterations. While opinions differ on the utility of rarefying sequence data for some purposes, it remains a valuable approach for studies such as ours aimed primarily at evaluating dissimilarity in community composition (McKnight et al., 2019; McMurdie & Holmes, 2014; Schloss, 2024; Weiss et al., 2017). For diet, the mOTU-by-sample matrix was iteratively rarefied to 3700 reads per sample (minimum reads per sample was 3739). For microbiome, ASV-by-sample matrix was iteratively rarefied to 5000 reads per sample, resulting in the removal of 2 microbiome samples with <5000 reads (Figure S4); this read threshold is comparable to those used in related studies of microbiome composition (e.g. Kartzinel et al., 2019) and was sufficient to capture the asymptote of ASV accumulation for most samples (Figure S4). Finally, both matrices were converted into proportions to yield relative read abundance (RRA) matrices. The final rarefied diet dataset contained 144 unique plant mOTUs from 79 faecal samples and the rarefied microbiome dataset contained 2892 unique bacterial ASVs from 84 faecal samples, with 75 faecal samples shared between the two datasets.

2.5 | Data analyses

Data were analysed and visualized in R v4.0.2 (R Core Team, 2020). We evaluated whether the five zebra subpopulations differed in the diversity of their diets and microbiomes, quantifying richness as the number of mOTUs/ASVs per sample and diversity using the Shannon-Weiner index in the *vegan* package (Oksanen et al., 2013). We then used analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) test to assess pairwise differences between the subpopulations (Figures 3c and 4c, Tables S2 and S5).

To interrogate differences in beta-diversity among samples and subpopulations, we calculated pairwise Bray–Curtis dissimilarity between all samples and visualized patterns with principal coordinate analysis (PCoA) for both diet (Figures 3a and S2) and microbiome (Figures 4a and S8) data using the 'vegan' package (Oksanen et al., 2013). We then conducted pairwise permutational multivariate analyses of variance (perMANOVA) to test for differences in diet and microbiome composition between the five subpopulations (Figures 3 and 4, Tables S3 and S6). We likewise performed analyses of multivariate homogeneity of group dispersions and tested for differences in the variability of diet and microbiome between the five subpopulations using Tukey HSD (Tables S3 and S6).

We used Bray–Curtis dissimilarity in part to facilitate direct comparison between the diet and microbiome data in this study and those in previous work, including from our study system (Brown et al., 2023; Kartzinel et al., 2019). To evaluate the sensitivity of these results in light of debates over the best approach to analysing microbiome composition data (e.g. Aitchison et al., 2000; McKnight et al., 2019; Reed et al., 2017; Regalado et al., 2020; Stothart et al., 2023; Weiss et al., 2017), we also calculated microbiome compositional dissimilarity between samples using three alternative approaches: Bray–Curtis dissimilarity, but with ASVs occurring in only one sample ('single-sample ASVs') removed, to assess whether Bray-Curtis results were driven by rare or low-abundance ASVs (Table S7); Aitchison distance, the Euclidean distance between samples after abundance data have been centered and log-ratio-transformed (Table S8), which is less sensitive to differences in presence-absence across samples than other distance metrics (Aitchison et al., 2000); and the weighted UniFrac distance metric (Lozupone & Knight, 2005), which incorporates phylogenetic relatedness of microbial taxa (Table S9). We repeated the statistical tests and visualizations described above using these metrics (Tables S6–S9 and Figures S5–S7).

To determine which taxa contributed most to differences between the diets and microbiomes of zebra subpopulations, we conducted indicator species analyses on both diet and microbiome datasets (Tables S4 and S10), using the *multipatt* function in the *indicspecies* package (De Caceres & Jansen, 2016). This function calculated the indicator value ('IndVal') association index for each mOTU/ASV and all five zebra subpopulations, determining the subpopulation with the highest IndVal for a given mOTU/ASV and evaluating the statistical significance of this association via permutation tests (with 999 permutations).

We then tested for diet-microbiome covariation. We first used linear regression to determine whether diet richness and diversity predicted microbiome richness and diversity respectively (Tables S11 and S12). We assessed correlations between diet and microbiome richness/diversity across all samples and for each subpopulation individually

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

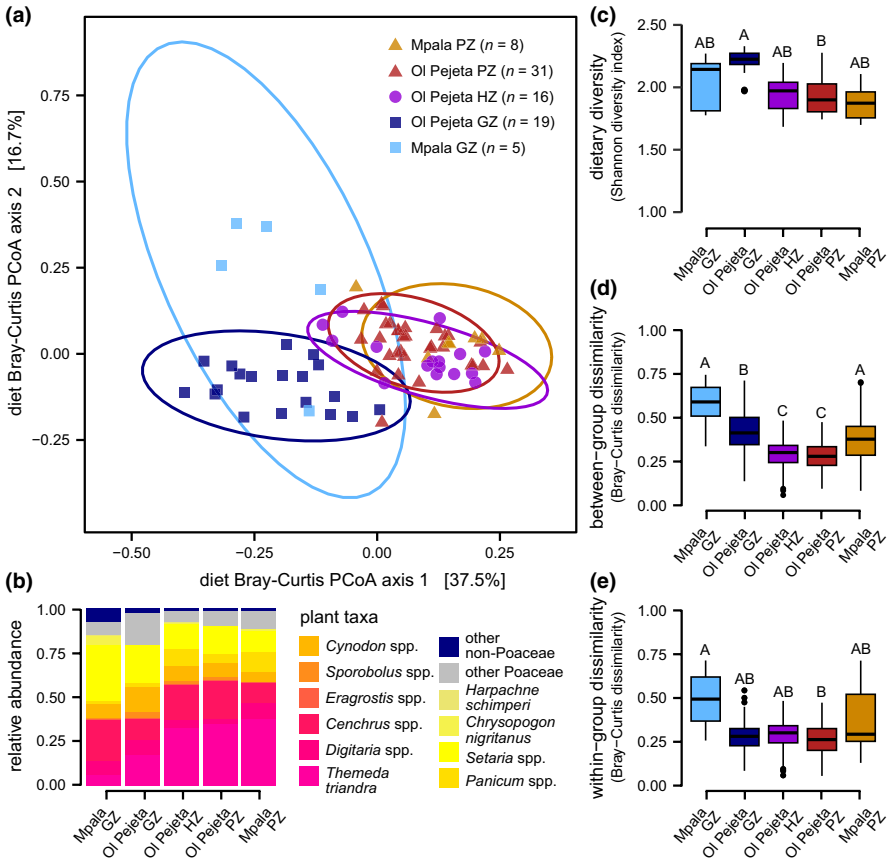

FIGURE 3 Diet composition and dissimilarity across zebra subpopulations and locations. (a) PCoA ordination plot showing Bray–Curtis dissimilarity in diet composition between samples. Each point represents a single faecal sample. Ellipses show 95% confidence intervals. Point and ellipse colours correspond to the five zebra subpopulations; point shapes correspond to the three zebra species. (b) Relative abundance of plant taxa in the diet of each zebra subpopulation, calculated by averaging the RRA for each plant taxon within subpopulations. (c) Dietary diversity (Shannon index) of each subpopulation. (d) Dietary dissimilarity of each subpopulation relative to hybrid zebra diets; the hybrid boxplot therefore reflects within-subpopulation dietary dissimilarity. (e) Within-subpopulation dietary dissimilarity, comparing each sample to others of the same species and location; thus, Ol Pejeta hybrid zebra box is identical to that in (d). In (c–e), black bars show median dissimilarity, coloured boxes show interquartile range (IQR), whiskers extend up to 1.5×IQR, dots are outliers. Letters denote statistically significant differences ($p < .05$); subpopulations with different letters are significantly different.

(Tables S11 and S12). To evaluate the correlation between diet and microbiome dissimilarities, we performed a Mantel test on our microbiome and diet Bray-Curtis dissimilarity matrices. To assess whether samples clustered similarly for diet and microbiome, we performed a symmetric Procrustes analysis, which evaluates the degree to which two ordinations can be aligned and superimposed on one another, with the diet and microbiome Bray-Curtis PCoAs. We then extracted the coordinates of each sample along the first axes of variation for both the diet and microbiome Bray-Curtis PCoAs and used linear regression to determine if diet PCoA values predicted microbiome PCoA values (Figure 5). As above, we assessed correlations between diet and microbiome PCoA values across all samples and for each subpopulation individually (Table S13).

To evaluate support for our four hypotheses (Figure 1b), we analysed microbiome composition as a function of species, location, sex and diet (represented by the values for each sample along the first axis of the diet PCoA; Figure S1), using marginal perMANOVA (Table 1). This approach assesses the marginal effect of each predictor by comparing models with and without that predictor, such that the ordering of predictors in the model call does not alter the estimated effect size or statistical significance. We also performed a stratified perMANOVA (stratified by subpopulation), which constrains the permutations in perMANOVA by a defined stratified variable to evaluate the effects of other predictors. We included diet and sex as predictors (Table S14), to evaluate their relative importance as predictors of within-subpopulation variation in microbiome composition.

3 | RESULTS

3.1 | Diet patterns

All five subpopulations ate >90% grass (family Poaceae; Figure 3b), consistent with previous work showing that zebras are among the most grass-specialist herbivores throughout Africa (Pansu

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

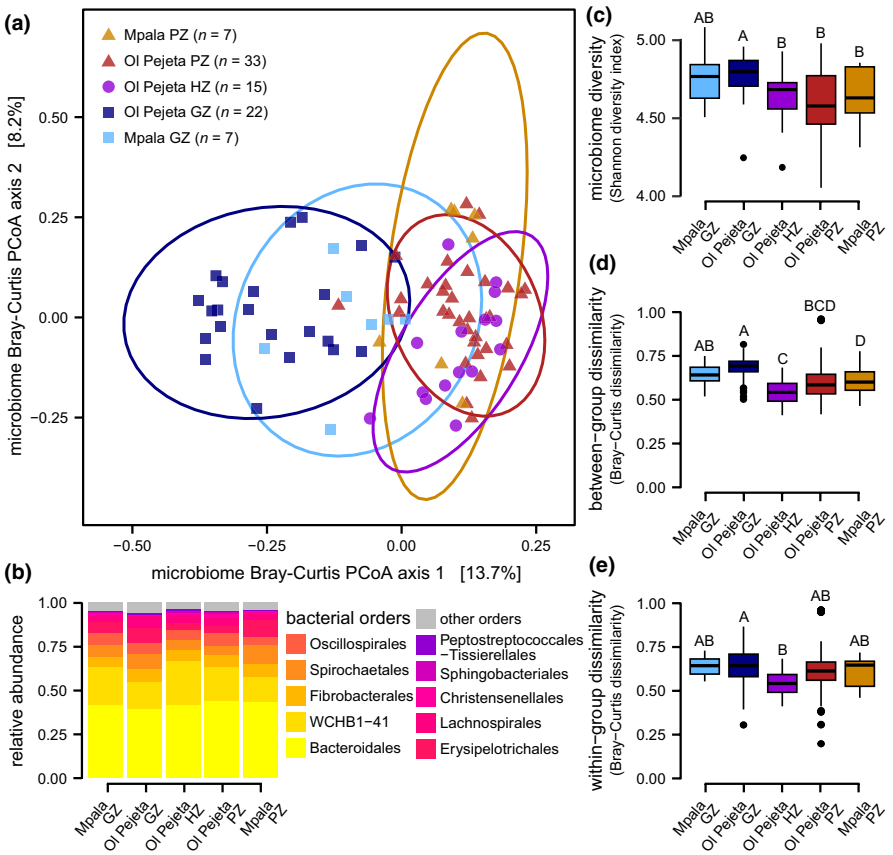

FIGURE 4 Microbiome composition and dissimilarity across subpopulations and locations. (a) PCoA ordination plot showing Bray–Curtis dissimilarity in microbiome composition between samples. Each point represents a single sample; ellipses represent 95% confidence intervals. Point and ellipse colours correspond to the five zebra subpopulations; point shapes correspond to the three zebra species. (b) Relative abundance of the ten most prevalent bacterial orders in zebra gut microbiomes, calculated by averaging the RRA of each bacterial order within each subpopulation. (c) Microbiome diversity (Shannon index) of each subpopulation. (d) Microbiome dissimilarity of each subpopulation relative to hybrid zebra; the hybrid boxplot therefore reflects within-subpopulation microbiome dissimilarity. (e) Within-subpopulation microbiome dissimilarity, comparing each sample to others from the same species and location; thus, Ol Pejeta hybrid zebra bar is identical to that in (d). In (c–e), black bars show median dissimilarity, boxes show interquartile range (IQR), whiskers extend up to 1.5×IQR, and dots are outliers. Letters denote statistically significant differences ($p < .05$); subpopulations with different letters are significantly different.

et al., 2022). The diversity of zebra diets exhibited little difference across the five subpopulations (Table S2), with no significant differences in richness and only one significant difference between subpopulations in Shannon diversity (Figure 3c). However, subpopulations differed in the contributions of particular plant mOTUs to their diets (Figure 3b), with variation in diet composition clustering first and foremost by species (Figure 3a): Grevy's zebra diets clustered together across locations, as did plains zebra diets. Hybrid zebra diets clustered with plains zebra from both locations (Figure 3a), being compositionally indistinguishable from those of Ol Pejeta plains but differing significantly from the other three subpopulations (Figure 3d and Table S3) despite including no unique plant mOTUs (Table S4). The diets of Mpala Grevy's and plains zebra did not differ significantly despite clustering separately in PCoA ordinations, likely reflecting limited statistical power arising from the small sample sizes in these subpopulations ($n = 5$ and 8; Figure S3). Ol Pejeta Grevy's had compositionally distinct diets (Figure 3d and Table S3), with considerably higher fractions of *Cynodon* spp. and *Sporobolus* spp. than the other subpopulations and two plant mOTUs that were unique to their diets (Figures 3b and S3, Table S4). The variability of hybrid diets was low, as was the variability of both Ol Pejeta Grevy's and plains diets; Mpala Grevy's had significantly more variable diets than those of Ol Pejeta plains (Figure 3e and Table S3).

3.2 | Microbiome patterns

Patterns of microbiome composition largely paralleled those of diet. Again, the ASV richness and diversity of zebra microbiomes differed little between the five subpopulations (Table S5), though Ol Pejeta Grevy's zebras did again have significantly more diverse microbiomes than Ol Pejeta plains (as with diet; Figure 3c) as well as Mpala plains and hybrids (Figure 4c). Based on Bray–Curtis dissimilarity, both with (Table S6 and Figure 4) and without (Table S7 and Figure S5) considering single-sample ASVs, and also based on Aitchison distance (Table S8 and Figure S6), the microbiomes of

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

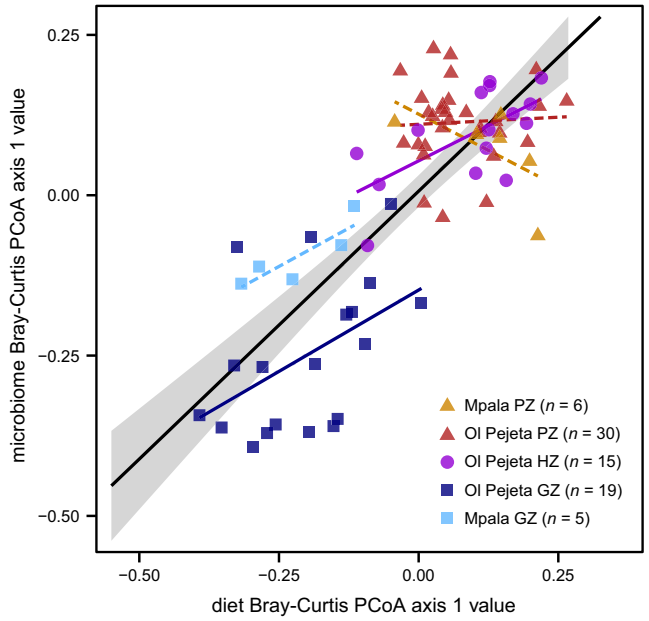

FIGURE 5 Correlation between diet and microbiome composition among and within species. Across all individuals, diet composition (approximated by values along diet Bray-Curtis PCoA axis 1) was correlated significantly with microbiome composition (approximated by values along microbiome Bray-Curtis PCoA axis 1). However, these correlations were variable and sometimes negligible within zebra subpopulations. Each point represents one faecal sample. Point colours correspond to zebra subpopulations; point shapes correspond to the three zebra species. Solid lines denote statistically significant relationships ($P \leq 0.05$) and dashed lines show non-significant relationships ($p > .05$). The grey shaded region shows the 95% confidence interval of the relationship between diet and microbiome composition across all individuals.

TABLE 1 Predictors of microbiome composition.

Predictor	df	Sum Sqs	R^2	Pseudo-*F*	*p*
Diet	1	0.260	.017	1.433	.072
Location	1	0.370	.025	2.040	.006
Sex	1	0.260	.017	1.435	.077
Species	2	0.880	.059	2.426	.001
Residual	66	11.967	.797		
Total	71	15.017	1.000		

Note: Results of marginal perMANOVA testing the relative contributions of location, species, sex, and diet (approximated by values along diet Bray-Curtis PCoA axis 1) to predicting the RRAs of microbial ASVs in zebra microbiomes.

hybrid zebra clustered with both of the plains zebra subpopulations in ordinations, and the microbiomes of the two Grevy's zebra subpopulations also clustered together, paralleling diet patterns. Statistically, however, microbiomes did not differ between subpopulations of the same species, no matter the distance metric used, in contrast to diet results (Tables S6–S9); plains zebra microbiomes were compositionally similar across locations, as were those of Grevy's zebra. Hybrid microbiomes also did not differ significantly from those of (maternal) Ol Pejeta plains zebra with any of the distance metrics (Tables S6–S9). Based on Bray–Curtis dissimilarity, hybrids differed significantly from the remaining three subpopulations, paralleling the pattern in diet composition (Figure 4d and Tables S6, S7). Using Aitchison distance, hybrid microbiomes did not differ from either subpopulation of plains zebra but still differed from both Grevy's subpopulations (Figure S6 and Table S8). Twenty-six bacterial ASVs were uniquely shared by hybrid and (maternal) plains zebra subpopulations, whereas hybrids only shared a single unique ASV with (paternal) Grevy's zebra subpopulations (Table S10). The within-group variability of hybrid microbiomes was also quite low relative to the other four subpopulations (Figure 4e), and significantly lower than the well-sampled Ol Pejeta Grevy's (again, regardless of the distance metric; Tables S6–S8).

When weighted UniFrac was used to quantify dissimilarity in microbiome composition between individuals, between-subpopulation differences in microbiome composition all but disappeared and all subpopulations clustered together (Table S9 and Figure S7): there were no statistical differences between any of the five subpopulations with regards to mean microbiome composition or compositional variability (Table S9), except that the composition of Ol Pejeta Grevy's microbiomes differed significantly from those of the hybrids (Figure S7). Accounting for phylogeny thus obscures differences in microbiome composition between subpopulations, indicating that differences are taxonomically superficial. Indeed, the proportional representation of bacterial taxa in the microbiome was similar across all five subpopulations (Figures 4b and S9–S11): microbiomes of all five subpopulations were dominated by the same five phyla (Bacteroidota, Firmicutes, Verrucomicrobiota, Fibrobacterota and Spirochaetota, together comprising >95% of all zebra microbiomes; Figure S9) and eight orders (Bacteroidales, WCHB1-41, Fibrobacterales, Oscillospirales, Spirochaetales, Erysipelotrichales, Lachnospirales and Christensenellales, together comprising >90% of the zebra microbiomes; Figures 4b and S10). Furthermore, of the 2892 total bacterial ASVs, all but 185 (6.4%) were shared across all subpopulations (Table S10).

3.3 | Diet-microbiome covariation

Dietary and microbiome richness were largely uncorrelated, across all samples and also within all but one subpopulation (Table S11). The lone exception was hybrids, which exhibited a significantly negative relationship (i.e. microbiome richness decreased with increasing dietary richness; Table S11). Dietary and microbiome diversity were entirely decoupled, overall and within each subpopulation (Table S12).

In contrast, we found strong compositional associations between zebra diets and microbiomes. Microbiome and diet dissimilarity matrices were significantly correlated (Mantel test; $n = 75$, $r = 0.366$, $p < .001$), and microbiome and diet Bray-Curtis PCoA ordinations were highly transposable (Procrustes test; $n = 75$, Sum of

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Squares = 0.636, Correlation = 0.604, $p < .001$). Diet and microbiome values along the first PCoA axes were significantly correlated across all samples (Figure 5 and Table S13), although the significance of this relationship did differ for each of the five subpopulations when evaluated individually: diet and microbiome PCoA values were not significantly correlated for either subpopulation of plains zebra, nor for Mpala Grevy's zebra, but were for hybrids and for Ol Pejeta Grevy's zebra (Figure 5 and Table S13).

Overall, we found that species explained the most variance in microbiome composition ($R^2 = 0.059$), followed by location ($R^2 = 0.025$), and then sex and diet ($R^2 = 0.017$ each) (Table 1). Sex and diet composition were both only marginally predictive of microbiome composition when controlling for these other predictors (Table 1), suggesting that the variation in diet and microbiome composition is driven by parallel factors in this system. However, when stratified by subpopulation (thus implicitly accounting for effects of species and location), we recovered the significance of diet as a predictor of microbiome composition, whereas sex was not significantly predictive (Table S14), suggesting that diet ultimately does have some predictive power for explaining within-subpopulation variability in microbiome composition.

4 | DISCUSSION

Here we use patterns of microbiome composition within a population of free-ranging hybrid zebras to evaluate evidence for four hypothesized mechanisms for microbiome fidelity within species (Table 2). Our results provide the strongest support for the maternal effects hypotheses (H_4): plains and Grevy's microbiomes were distinct from one another but were compositionally similar across locations, and hybrid microbiomes were compositionally indistinguishable from plains zebra microbiomes, the maternal species. Furthermore, hybrid microbiomes were less variable than those of plains zebra, suggesting that hybrid microbiomes effectively represent a nested subset of plains zebra microbiomes. We also find some support for the hypothesis that diet regulates microbiome composition (H_1): diet and microbiome dissimilarity matrices were significantly correlated, and ordinations were highly transposable. However, diets of the same species differed significantly between sampling locations, whereas microbiomes of the same species did not. And although diet and microbiome values along the first PCoA axes were significantly correlated across all samples, correlations varied within individual subpopulations. The richness and diversity of zebra diets and microbiomes were largely decoupled. We find limited support for the morphophysiological filtering hypothesis (H_3): hybrids did not have intermediate microbiomes, as would be predicted from their intermediate morphology (Cordingley et al., 2009; Schieltz & Rubenstein, 2015). Our results also reflect weak support for the environmental sourcing hypothesis (H_2): microbiomes of the same species were compositionally indistinguishable across locations, and sex had limited predictive power for explaining microbiome composition.

4.1 | H_1: Diet regulates microbiome composition

We found that hybrid diets were indistinguishable from plains zebra diets, despite the genetic contribution from Grevy's zebra (Cordingley et al., 2009; Schieltz & Rubenstein, 2015). Some aspects of diet are heritable and exhibit phylogenetic signal (Abraham et al., 2022; Codron et al., 2019; Kartzinel et al., 2019). Also, hybrids are of intermediate body size relative to their parent species (Cordingley et al., 2009; Schieltz & Rubenstein, 2015), and body size plays a key role in herbivore foraging behaviour and diet composition (Abraham et al., 2022; Daskin et al., 2023; Demment & Van Soest, 1985; Hopcraft et al., 2010; Pansu et al., 2022). For both of these reasons, hybrids might be expected to exhibit intermediate diets to their parent species. Instead, we found that hybrid diets were indistinguishable from plains zebra diets where they co-occurred. As hybrids are raised exclusively in the plains zebra society (Schieltz & Rubenstein, 2015), the similarity of hybrid diets to those of plains zebra suggests that at least some aspects of their foraging behaviour are learned, rather than genetically determined. Learning and memory are indeed known to play a role in other components of herbivorous mammal foraging, such as migration (Jesmer et al., 2018; Mueller et al., 2013). Generally, an herbivore's foraging behaviour may be shaped by the social influences of other herbivores where they are raised and learn to forage.

Differences in diet between zebra subpopulations appear to have ramifications for their microbiomes: several lines of evidence were consistent with H_1, that diet regulates microbiome composition (Table 2). Consistent with predictions of H_1, we find that zebra microbiome patterns broadly paralleled diet patterns. Diet and microbiome dissimilarity matrices were significantly correlated and PCoA ordinations were highly transposable; diets and microbiomes of hybrids both clustered with those of Ol Pejeta plains zebra; diet was a significant predictors of microbiome composition in a perMANOVA stratified by subpopulation (Table S14); and diet and microbiome values along the first PCoA axes of were significantly correlated when all samples were considered (Figure 5). However, inconsistent with H_1, diets of the same species differed significantly across sites (Figure 3d), whereas microbiomes of the same species did not (Figure 4d); diet was not significantly predictive of microbiome composition in a marginal perMANOVA (Table 1); dietary richness and diversity were largely uncoupled from microbiome richness and diversity, except within hybrids (Tables S11 and S12); and correlations between diet and microbiome values along the first PCoA axes varied for individual subpopulations, being non-significant for both subpopulations of plains zebra and for Mpala Grevy's zebra (Figure 5).

That diet plays some role in regulating microbiome composition accords with a large body of research documenting correlations between diet and microbiome in mammals at various scales: across species and lineages (Muegge et al., 2011; Sanders et al., 2015), within and among co-occurring populations (Bergmann et al., 2015; Kartzinel et al., 2019), and within individuals (David et al., 2014). However, the divergences observed here between diet and microbiome patterns suggest that diet alone cannot explain microbiome

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Summary of evidence in relation to each hypothesis.

Hypothesis	Predictions	Evidence consistent with prediction	Evidence inconsistent with prediction	Relevant tables/figures
H_1: diet regulates microbiome composition	Diet composition and microbiome composition should be correlated	Both hybrid diets and microbiomes clustered with Ol Pejeta PZ	Species' diets differed across locations, but micr-obiomes did not	Figures 3d and 4d
		Diet significantly predicted micro-biome composition in stratified perMANOVA	Diet was not predictive of micro-biome composition in marginal perMANOVA	Tables 1 and S5
		Diet and micro-biome PCoA values were significantly correlated across all samples	Correlations between diet and microbiome PCoA values varied by subpopulation	Figure 5
		Diet/microbiome dissimilarity matrices were corr-elated, and PCoA ordinations were highly transposable	Dietary richness and diversity were largely uncoupled from microbiome richness and diversity	Figures 3a and 4a; Tables S11–S13
H_2: microbiomes are a result of shared environmental influences	Location should be a primary axis of microbiome differentiation, as should sex for GZ and HZ (due to their sex-specific landscape use)	Location signific-antly predicted microbiome composition in marginal perMANOVA	Sex did not predict microbiome composition in either stratified or marginal perMANOVAs	Tables 1 and S5
		Mpala zebras shared 34 unique ASVs (though no ASVs were unique to Ol Pejeta zebras)	Microbiomes of the same species did not differ significantly between locations	Table S10; Figure 4d
			In microbiome PCoA, species clustered together across sites (rather than clustering by site) and did not cluster by sex	Figures 4a and S8C
H_3: microbiomes are filtered by gut host anatomy	Hybrids, morphologically intermediate to their parent species, should have compositionally intermediate microbiomes. Also, microbiomes should be more variable than those of their parents, due to greater hybrid phenotypic variability		Hybrid micro-biomes were largely indistinguishable from Ol Pejeta PZ, not intermediate to PZ and GZ	Figure 4d
			Hybrid micro-biomes were significantly less variable, not more variable, than parent species'	Table S11; Figure 4e
H_4: microbiome composition is determined by maternal effects	Microbiomes of the same species should cluster across locations. Hybrid microbiomes should cluster with their maternal parent species, PZ. Also, hybrid microbiomes should be less variable than those of their parents, as they effectively represent a subset of PZ microbiomes	Hybrid micro-biomes were statistically indistinguishable from those of co-occurring maternal parent species, PZ (no matter the distance metric)	Hybrid microbiomes differed from those of Mpala PZ in terms of Bray–Curtis dissimilarity (but did not in terms of Aitchison distance)	Figure 4a,d
		Microbiomes did not significantly differ for the same species across locations		Figure 4a,d
		Hybrid micro-biomes were less variable than those of co-occurring parent species		Figure 4e
		Hybrids shared 26 unique bacterial ASVs with PZ subpopulations, whereas hybrids only shared one ASV with GZ		Table S10

Note: Results were broadly congruent with the predictions of H_4 (microbiomes are derived from maternal effects) and supported some predictions of H_1 (diet regulates microbiome composition). We recover weak support for H_2 (microbiomes are a product of shared environment) and H_3 (microbiomes are filtered by host gut anatomy).

13652994x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

fidelity within species – diet may therefore be a proximate, rather than ultimate, driver of microbiome composition. For instance, recent research has demonstrated that the microbiome may influence host foraging behaviour, altering the food preferences of the host (Trevelline & Kohl, 2022), such that microbiome composition may modulate diet composition, not vice versa. Microbiome composition might initially arise via some other mechanism but might then be reinforced or stabilized by diet-microbiome feedbacks. Alternatively, diet and microbiome composition may both be determined by parallel factors. In the context of our study system, hybrid diets and microbiomes may both be influenced by the fact that hybrids are raised with plains zebras, their maternal species (see H_4: *microbiome composition is determined by maternal effects* below), which may account for the diet-microbiome covariation we recover here. Further research is needed to test whether impacts of diet on microbiome are direct, for example, due to effects of digesting particular foods on gut hospitability for particular microbes (Hammer et al., 2019; Kohl et al., 2014; Schluter & Foster, 2012; Zhang et al., 2016), or indirect, each responding to other forces that generally operate in parallel on diet and microbiome composition within wild populations.

4.2 | H_2: Microbiomes are a result of shared environment

We find limited support for H_2, that shared environmental exposures contribute to microbiome composition. Consistent with H_2, we found that location was a significant predictor of microbiome composition in a marginal perMANOVA, second in predictive power only to species. Also, the two zebra subpopulations from Mpala shared 34 unique ASVs (though no ASVs were uniquely shared across the three Ol Pejeta subpopulations; Table S10). However, inconsistent with H_2, we found that sex was not significantly predictive of microbiome composition (in contrast to expectations under H_2, since male and female Grevy's and hybrids differ in their landscape use; Schieltz & Rubenstein, 2015), though we acknowledge that we had limited power to detect sex differences due to sample sizes constraints (Table S1). Also, microbiomes of co-occurring subpopulations were significantly different from one another (Figure 4d). Lastly, in PCoA ordinations of microbiome composition, microbiomes did not cluster by location, but rather by species (Figure 4a), and there was also no discernable clustering by sex (Figure S8).

Some studies have found that environmental influences do affect microbiome composition (Couch et al., 2020; Grieneisen et al., 2019; Metcalf et al., 2017; Tasnim et al., 2017; Yatsunenko et al., 2012). For example, in a study on hybrid baboons, soil type was shown to predict microbiome similarity across hybrid subpopulations, rather than genetic factors (Grieneisen et al., 2019). However, such studies rarely control explicitly for the effects of diet. Indeed, in the same study on hybrid baboons, the authors cite turnover in food availability across soil types as a likely mechanism for the observed effect of soil composition on the microbiome (Grieneisen et al., 2019). Relatedly, differences in the microbiomes of captive and wild animals, a classic example of the profound impact that environmental context can have on microbiome composition (McKenzie et al., 2017; Metcalf et al., 2017), can at least partially be accounted for by the fact that captive animals are fed radically different, highly artificial diets relative to their wild counterparts (Metcalf et al., 2017). As such, effects of environmental influences may be largely mediated through other variables, especially diet, hence the limited support we recover for H_2 when accounting for these other factors.

4.3 | H_3: Microbiomes are filtered by host gut anatomy

We find minimal support for H_3, that host gut anatomy acts as a filter on microbiome composition. Under H_3, the hybrid zebras, which are morphologically intermediate to their parent species (Cordingley et al., 2009), would be expected to have compositionally intermediate microbiomes. Instead, we found that the microbiomes of hybrids were largely indistinguishable from Ol Pejeta plains zebra (Figure 4d). Furthermore, hybrid microbiomes were significantly less variable than those of their parent species (Figure 4e); the generally greater phenotypic variability of hybrids relative to parent species (Harrison, 1990; Russell, 1941) would suggest that hybrid microbiomes should be more, not less, variable than those of their parent species.

That we find limited support for H_3 contrasts with other research suggesting that host morphology and physiology play a foremost role in determining the composition of the gut microbiome (Amato et al., 2019; Godon et al., 2016; Song et al., 2020). However, such analyses often deal with large taxonomic scales and extreme shifts in host morphophysiology (Amato et al., 2019; Godon et al., 2016; Song et al., 2020). In this system, individuals differ by at most ~100 kg in body size, such that morphophysiological differences may be too subtle to overwhelm other drivers of microbiome composition. Relatedly, all five zebra subpopulations ate predominantly grass, such that their microbiomes are likely highly constrained by their need to extract nutrition from this recalcitrant forage. Indeed, this functional constraint may account, at least in part, for the lack of differentiation between the subpopulations when assessed with weighted UniFrac distance. Bacterial function is highly phylogenetically conserved (Martiny et al., 2013; Philippot et al., 2010), and all zebras likely require microbiomes with relatively similar phylogenetic composition in order to adequately process their grass-rich diets (Kartzinel et al., 2019; Muegge et al., 2011). Furthermore, though hybrids are intermediate with regards to body size and presumably gut size (Duque-Correa et al., 2021), other aspects of physiology may exhibit more complex patterns of inheritance, such that the gut environment of hybrids may not be intermediate in all regards. Alternatively, the effects of morphophysiology on the microbiome

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

may be mediated by other factors within this hybrid zebra system, as with environmental influences. As described above, diet is influenced by body size (Daskin et al., 2023; Demment & Van Soest, 1985; Hopcraft et al., 2010; Pansu et al., 2022), so diet may capture any effect of morphology on the microbiome. Accounting for other factors influenced by morphophysiology – in this case, diet – may therefore obscure any effect that morphology ultimately has on the microbiome.

4.4 | H_4: Microbiome composition is determined by maternal effects

We find largely consistent support for H_4, that microbiome composition is determined by maternal effects (Table 2). We found that hybrid zebra microbiomes were compositionally indistinguishable from those of co-occurring plains zebra, their maternal parent species, and microbiomes of the same species were similar across locations (Figure 4a) (no matter the distance metric used). Hybrids uniquely shared 26 bacterial ASVs with (maternal) plains zebra subpopulations (as opposed to the single ASV shared between hybrids and any Grevy's population; Table S10). Moreover, hybrid microbiomes were less variable than those of other subpopulations with which they co-occurred (Figure 4e), indicating that hybrid microbiomes effectively represent a nested subset of plains zebra microbiomes. Somewhat inconsistent with H_4, we found that hybrid microbiomes did differ from those of Mpala plains zebra when measured with Bray–Curtis dissimilarity (but not with Aitchison distance). However, this may reflect the fact that the two study locations are isolated, such that slight drift may have occurred in microbiome composition between locations.

Maternal effects on microbiome composition are thought to occur via direct transmission of microbes from mother to offspring. During birth, mammals are exposed to the microbiome of their mother's birth canal and, subsequently, to their mother's skin microbiome while nursing (Bäckhed et al., 2005; Blaser & Dominguez-Bello, 2016; Koenig et al., 2011; Tanaka & Nakayama, 2017). These maternal microbes are thought to seed the microbiomes of their offspring (Blaser & Dominguez-Bello, 2016; Rosshart et al., 2019). However, novel microbes are constantly introduced to the microbiome throughout an organism's life (Hammer et al., 2019; Zhang et al., 2016). If the microbiome is sourced from the mother during parturition and nursing, then some intrinsic mechanism (i.e. priming of the immune system to specific microbes; Burr et al., 2020; Rosshart et al., 2019; Shi et al., 2017) must preserve this initial microbial community in the face of constant introductions of new microbes from the environment (Bäckhed et al., 2005; D'Argenio, 2018; Zhang et al., 2016). Indeed, the majority (but not all) of the zebra included in this study were adults (Figure S8), and we found that hybrid microbiomes still resembled those of the maternal parent species. Further research on how hosts modulate microbiome composition over the course of their lives would illuminate whether maternal effects do in fact account for microbiome fidelity within species.

4.5 | Limitations

While our approach provides new insights into the processes underlying microbiome assembly, it is not without limitations. All the samples were collected within a single season, which prevents us from evaluating the stability of these patterns over time. Indeed, seasonality is known to play a large role in microbiome assembly (Bergmann et al., 2015; Kartzinel et al., 2019), and future work should therefore include longitudinal sampling of these subpopulations. Also, due to the scarcity of the endangered Grevy's zebra, and especially hybrid zebras, of which only ~20 are known to occur in the wild, sample sizes of certain subpopulations were necessarily small, such that we had limited power to detect compositional differences between the diets and microbiomes of some groups (Table S1). Finally, we used 16S amplicon sequencing to characterize microbiome composition, which targets the bacterial component of the microbiome; it is well-established that fungi, viruses and phages also represent a significant component of the gut microbiome and they may exhibit different patterns of transmission (Dearing & Kohl, 2017; Ley et al., 2008; Neish, 2009). We amplified the 16S rRNA V4 region so that our results would be directly comparable with other studies (e.g. Kartzinel et al., 2019; Metcalf et al., 2017). However, targeting non-bacterial genes and other bacterial genes that evolve faster may provide greater resolution into microbiome differences (Moeller et al., 2016; Ogier et al., 2019), though this is an area of active research development (Ogier et al., 2019). Likewise, 16S rRNA sequencing often precludes drawing precise functional conclusions about the microbiome, as analyses are limited to only compositional, taxonomic associations. Still, 16S rRNA sequencing can be surprisingly predictive of whole genome sequencing of bacterial microbiomes (Regalado et al., 2020; Stothart et al., 2023), and taxonomic information does provide some insight into microbiome function (Martiny et al., 2013; Muegge et al., 2011; Philippot et al., 2010). Future work using other bacterial amplicons, shotgun or whole genome sequencing, and including non-bacterial components of the microbiome will provide further insights into microbiome assembly and function.

4.6 | Conclusions

Overall, we find the strongest support for maternal effects (H_4) driving microbiome composition, we find considerable support for the role of diet (H_1) in structuring microbiome composition, and we find limited support for a strong direct role of environment (H_2) or morphophysiological filtering (H_3) on the gut microbiome. Though these hybrid zebras allow for greater resolution in evaluating the relative support for these four hypotheses, this hybrid subpopulation does not allow us to fully isolate these four mechanisms. For instance, the diets and microbiomes of hybrids both clustered with those of plains zebra, their maternal species, a pattern that simultaneously supports H_1 and H_4. Here, we did not know the diets of the hybrid zebra a priori, as they had not been characterized prior to this study. But studying a hybrid subpopulation where the diets of hybrids are known to

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

differ from their parent species might fully allow for the parsing of these hypotheses. By carefully designing studies that isolate genetic, behavioural, and environmental factors and weighing evidence for and against hypotheses, as we have done here, we can build a more complete picture of the relative contributions of these four mechanisms to conserving microbiome composition within species.

AUTHOR CONTRIBUTIONS

AEM and BL conceived of the project and designed the sampling approach, with input from DIR and JFA. AEM and BL led faecal sampling in the field, with help from RW, MM, and PML to find and identify hybrids. JOA, AEM and BL performed lab work, with technical advice from LFP, LPH, and MYD. Lab work was conducted at the Mpala genomics facility in Kenya and at RMP's laboratory at Princeton University. JOA led bioinformatics and data analyses, with input from LPH, MYD, and RMP. JOA designed figures and drafted the manuscript, with input from RMP and other co-authors. All co-authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

Research was conducted in accordance with the Kenya National Commission for Science, Technology & Innovation permit #402036 to DIR.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

FUNDING INFORMATION

This work was supported by NSF DEB-2225088 and the High Meadows Environmental Institute at Princeton University. JOA is supported by the NSF Graduate Research Fellowship (Fellow ID: 2019256075).

DATA AVAILABILITY STATEMENT

Raw and filtered sequencing data, R code and all files necessary to reproduce these analyses are deposited in Dryad Digital Repository (DOI: https://doi.org/10.5061/dryad.0rxwdbs7c).

ORCID

Joel O. Abraham https://orcid.org/0000-0002-4704-1593
Bing Lin https://orcid.org/0000-0002-5905-9512
Margaret Y. Demmel https://orcid.org/0000-0001-5754-7638
Luisa F. Pallares https://orcid.org/0000-0001-6547-1901
Robert M. Pringle https://orcid.org/0000-0001-7487-5393
Daniel I. Rubenstein https://orcid.org/0000-0002-3747-9946

REFERENCES

Abraham, J. O., Upham, N. S., Damian-Serrano, A., & Jesmer, B. R. (2022). Evolutionary causes and consequences of ungulate migration. *Nature Ecology & Evolution*, *1–9*, 998–1006. https://doi.org/10.1038/s41559-022-01749-4

Aitchison, J., Barcelo-Vidal, C., Martın-Fernandez, J. A., & Pawlowsky-Glahn, V. (2000). Logratio analysis and compositional distance. *Mathematical Geology*, *32*, 271–275.

Alston, J. M., Reed, C. G., Khasoha, L. M., Brown, B. R. P., Busienei, G., Carlson, N., Coverdale, T. C., Dudenhoeffer, M., Dyck, M. A., Ekeno, J., Hassan, A. A., Hohbein, R., Jakopak, R. P., Kimiti, B., Kurukura, S., Lokeny, P., Louthan, A. M., Musila, S., Musili, P. M., … Goheen, J. R. (2022). Ecological consequences of large herbivore exclusion in an African savanna: 12 years of data from the UHURU experiment. *Ecology*, *103*(4), e3649. https://doi.org/10.1002/ecy.3649

Amato, K. R., Sanders, G., J., Song, S. J., Nute, M., Metcalf, J. L., Thompson, L. R., Morton, J. T., Amir, A., McKenzie, V. J., Humphrey, G., Gogul, G., Gaffney, J., Baden, A. L., Britton, G. A. O., Cuozzo, F. P., Di Fiore, A., Dominy, N. J., Goldberg, T. L., … Leigh, S. R. (2019). Evolutionary trends in host physiology outweigh dietary niche in structuring primate gut microbiomes. *The ISME Journal*, *13*(3), Article 3., 576–587. https://doi.org/10.1038/s41396-018-0175-0

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science*, *307*(5717), 1915–1920. https://doi.org/10.1126/science.1104816

Baniel, A., Petrullo, L., Mercer, A., Reitsema, L., Sams, S., Beehner, J. C., Bergman, T. J., Snyder-Mackler, N., & Lu, A. (2022). Maternal effects on early-life gut microbiota maturation in a wild nonhuman primate. *Current Biology*, *32*(20), 4508–4520.e6. https://doi.org/10.1016/j.cub.2022.08.037

Bergmann, G. T., Craine, J. M., Robeson, M. S., & Fierer, N. (2015). Seasonal shifts in diet and gut microbiota of the American bison (*Bison bison*). *PLoS One*, *10*(11), e0142409. https://doi.org/10.1371/journal.pone.0142409

Bergström, A., Skov, T. H., Bahl, M. I., Roager, H. M., Christensen, L. B., Ejlerskov, K. T., Mølgaard, C., Michaelsen, K. F., & Licht, T. R. (2014). Establishment of intestinal microbiota during early life: A longitudinal, explorative study of a large cohort of Danish infants. *Applied and Environmental Microbiology*, *80*(9), 2889–2900. https://doi.org/10.1128/AEM.00342-14

Blaser, M. J., & Dominguez-Bello, M. G. (2016). The human microbiome before birth. *Cell Host & Microbe*, *20*(5), 558–560. https://doi.org/10.1016/j.chom.2016.10.014

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), Article 8., 852–857. https://doi.org/10.1038/s41587-019-0209-9

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). Obitools: A unix-inspired software package for DNA metabarcoding. *Molecular Ecology Resources*, *16*(1), 176–182. https://doi.org/10.1111/1755-0998.12428

Brooks, A. W., Kohl, K. D., Brucker, R. M., van Opstal, E. J., & Bordenstein, S. R. (2016). Phylosymbiosis: Relationships and functional effects of microbial communities across host evolutionary history. *PLoS Biology*, *14*(11), e2000225. https://doi.org/10.1371/journal.pbio.2000225

Brown, B. R. P., Goheen, J. R., Newsome, S. D., Pringle, R. M., Palmer, T. M., Khasoha, L. M., & Kartzinel, T. R. (2023). Host phylogeny and functional traits differentiate gut microbiomes in a diverse natural community of small mammals. *Molecular Ecology*, *32*(9), 2320–2334. https://doi.org/10.1111/mec.16874

Burr, A. H. P., Bhattacharjee, A., & Hand, T. W. (2020). Nutritional modulation of the microbiome and immune response. *Journal of Immunology*, *205*(6), 1479–1487. https://doi.org/10.4049/jimmunol.2000419

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Codron, D., Hofmann, R. R., & Clauss, M. (2019). Morphological and physiological adaptations for browsing and grazing. In I. J. Gordon & H. H. T. Prins (Eds.), *The ecology of browsing and grazing II* (pp. 81–125). Springer International Publishing. https://doi.org/10.1007/978-3-030-25865-8_4

Cordingley, J. E., Sundaresan, S. R., Fischhoff, I. R., Shapiro, B., Ruskey, J., & Rubenstein, D. I. (2009). Is the endangered Grevy's zebra threatened by hybridization? *Animal Conservation*, *12*(6), 505–513. https://doi.org/10.1111/j.1469-1795.2009.00294.x

Costa, M. C., & Weese, J. S. (2012). The equine intestinal microbiome. *Animal Health Research Reviews*, *13*(1), 121–128. https://doi.org/10.1017/S1466252312000035

Couch, C. E., Arnold, H. K., Crowhurst, R. S., Jolles, A. E., Sharpton, T. J., Witczak, M. F., Epps, C. W., & Beechler, B. R. (2020). Bighorn sheep gut microbiomes associate with genetic and spatial structure across a metapopulation. *Scientific Reports*, *10*(1), 6582. https://doi.org/10.1038/s41598-020-63401-0

D'Argenio, V. (2018). Human microbiome acquisition and Bioinformatic challenges in metagenomic studies. *International Journal of Molecular Sciences*, *19*(2), 383. https://doi.org/10.3390/ijms19020383

Daskin, J. H., Becker, J. A., Kartzinel, T. R., Potter, A. B., Walker, R. H., Eriksson, F. A. A., Buoncore, C., Getraer, A., Long, R. A., & Pringle, R. M. (2023). Allometry of behavior and niche differentiation among congeneric African antelopes. *Ecological Monographs*, *93*(1), e1549. https://doi.org/10.1002/ecm.1549

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563. https://doi.org/10.1038/nature12820

De Caceres, M., & Jansen, F. (2016). *Package "indicspecies"* [computer software].

Dearing, M. D., & Kohl, K. D. (2017). Beyond fermentation: Other important services provided to endothermic herbivores by their gut microbiota. *Integrative and Comparative Biology*, *57*(4), 723–731. https://doi.org/10.1093/icb/icx020

Demment, M. W., & Van Soest, P. J. (1985). A nutritional explanation for body-size patterns of ruminant and nonruminant herbivores. *The American Naturalist*, *125*(5), 641–672. https://doi.org/10.1086/284369

Duque-Correa, M. J., Codron, D., Meloro, C., McGrosky, A., Schiffmann, C., Edwards, M. S., & Clauss, M. (2021). Mammalian intestinal allometry, phylogeny, trophic level and climate. *Proceedings of the Royal Society B: Biological Sciences*, *288*, 20202888. https://doi.org/10.1098/rspb.2020.2888

Elith, J., & Leathwick, J. R. (2009). Species distribution models: Ecological explanation and prediction across space and time. *Annual Review of Ecology, Evolution, and Systematics*, *40*(1), 677–697. https://doi.org/10.1146/annurev.ecolsys.110308.120159

Ficetola, G. F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., Taberlet, P., & Pompanon, F. (2010). An in silico approach for the evaluation of DNA barcodes. *BMC Genomics*, *11*(1), 434. https://doi.org/10.1186/1471-2164-11-434

Fischhoff, I. R., Sundaresan, S. R., Cordingley, J., & Rubenstein, D. (2007). Habitat use and movements of plains zebra (*Equus burchelli*) in response to predation danger from lions. *Behavioral Ecology*, *18*(4), 725–729. https://doi.org/10.1093/beheco/arm036

Georgiadis, N. J., Ihwagi, F., Olwero, J. G. N., & Romañach, S. S. (2007). Savanna herbivore dynamics in a livestock-dominated landscape. II: Ecological, conservation, and management implications of predator restoration. *Biological Conservation*, *137*(3), 473–483. https://doi.org/10.1016/j.biocon.2007.03.006

Gill, B. A., Musili, P. M., Kurukura, S., Hassan, A. A., Goheen, J. R., Kress, W. J., Kuzmina, M., Pringle, R. M., & Kartzinel, T. R. (2019). Plant DNA-barcode library and community phylogeny for a semi-arid east African savanna. *Molecular Ecology Resources*, *19*(4), 838–846. https://doi.org/10.1111/1755-0998.13001

Godon, J.-J., Arulazhagan, P., Steyer, J.-P., & Hamelin, J. (2016). Vertebrate bacterial gut diversity: Size also matters. *BMC Ecology*, *16*(1), 12. https://doi.org/10.1186/s12898-016-0071-2

Goheen, J. R., Palmer, T. M., Charles, G. K., Helgen, K. M., Kinyua, S. N., Maclean, J. E., Turner, B. L., Young, H. S., & Pringle, R. M. (2013). Piecewise disassembly of a large-herbivore community across a rainfall gradient: The UHURU experiment. *PLoS One*, *8*(2), e55192. https://doi.org/10.1371/journal.pone.0055192

Grieneisen, L. E., Charpentier, M. J. E., Alberts, S. C., Blekhman, R., Bradburd, G., Tung, J., & Archie, E. A. (2019). Genes, geology and germs: Gut microbiota across a primate hybrid zone are explained by site soil properties, not host species. *Proceedings of the Royal Society B: Biological Sciences*, *286*, 20190431. https://doi.org/10.1098/rspb.2019.0431

Groussin, M., Mazel, F., Sanders, J. G., Smillie, C. S., Lavergne, S., Thuiller, W., & Alm, E. J. (2017). Unraveling the processes shaping mammalian gut microbiomes over evolutionary time. *Nature Communications*, *8*(1), 14319. https://doi.org/10.1038/ncomms14319

Hammer, T. J., Sanders, J. G., & Fierer, N. (2019). Not all animals need a microbiome. *FEMS Microbiology Letters*, *366*(10), fnz117. https://doi.org/10.1093/femsle/fnz117

Harrison, R. G. (1990). Hybrid zones: Windows on evolutionary process. *Oxford Surveys in Evolutionary Biology*, *7*, 69–128.

Hopcraft, J. G. C., Olff, H., & Sinclair, A. R. E. (2010). Herbivores, resources and risks: Alternating regulation along primary environmental gradients in savannas. *Trends in Ecology & Evolution*, *25*(2), 119–128. https://doi.org/10.1016/j.tree.2009.08.001

Jesmer, B. R., Merkle, J. A., Goheen, J. R., Aikens, E. O., Beck, J. L., Courtemanch, A. B., Hurley, M. A., McWhirter, D. E., Miyasaki, H. M., Monteith, K. L., & Kauffman, M. J. (2018). Is ungulate migration culturally transmitted? Evidence of social learning from translocated animals. *Science*, *361*(6406), 1023–1025. https://doi.org/10.1126/science.aat0985

Kartzinel, T. R., Chen, P. A., Coverdale, T. C., Erickson, D. L., Kress, W. J., Kuzmina, M. L., Rubenstein, D. I., Wang, W., & Pringle, R. M. (2015). DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. *Proceedings of the National Academy of Sciences*, *112*(26), 8019–8024. https://doi.org/10.1073/pnas.1503283112

Kartzinel, T. R., Hsing, J. C., Musili, P. M., Brown, B. R. P., & Pringle, R. M. (2019). Covariation of diet and gut microbiome in African megafauna. *Proceedings of the National Academy of Sciences*, *116*(47), 23588–23593. https://doi.org/10.1073/pnas.1905666116

Kihwele, E. S., Mchomvu, V., Owen-Smith, N., Hetem, R. S., Hutchinson, M. C., Potter, A. B., Olff, H., & Veldhuis, M. P. (2020). Quantifying water requirements of African ungulates through a combination of functional traits. *Ecological Monographs*, *90*(2), e01404. https://doi.org/10.1002/ecm.1404

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., Angenent, L. T., & Ley, R. E. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences*, *108*(supplement_1), 4578–4585. https://doi.org/10.1073/pnas.1000081107

Kohl, K. D., Weiss, R. B., Cox, J., Dale, C., & Denise Dearing, M. (2014). Gut microbes of mammalian herbivores facilitate intake of plant toxins. *Ecology Letters*, *17*(10), 1238–1246. https://doi.org/10.1111/ele.12329

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., Schlegel, M. L., Tucker, T. A., Schrenzel, M. D., Knight, R., & Gordon, J. I. (2008). Evolution of mammals and their gut microbes. *Science*, *320*(5883), 1647–1651. https://doi.org/10.1126/science.1155725

Li, Z., Wright, A.-D. G., Si, H., Wang, X., Qian, W., Zhang, Z., & Li, G. (2016). Changes in the rumen microbiome and metabolites

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

reveal the effect of host genetics on hybrid crosses. *Environmental Microbiology Reports*, *8*(6), 1016–1023. https://doi.org/10.1111/1758-2229.12482

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Martiny, A. C., Treseder, K., & Pusch, G. (2013). Phylogenetic conservatism of functional traits in microorganisms. *The ISME Journal*, *7*(4), 830–838. https://doi.org/10.1038/ismej.2012.160

McKenzie, V. J., Song, S. J., Delsuc, F., Prest, T. L., Oliverio, A. M., Korpita, T. M., Alexiev, A., Amato, K. R., Metcalf, J. L., Kowalewski, M., Avenant, N. L., Link, A., Di Fiore, A., Seguin-Orlando, A., Feh, C., Orlando, L., Mendelson, J. R., Sanders, J., & Knight, R. (2017). The effects of captivity on the mammalian gut microbiome. *Integrative and Comparative Biology*, *57*(4), 690–704. https://doi.org/10.1093/icb/icx090

McKinney, C. A., Oliveira, B. C. M., Bedenice, D., Paradis, M.-R., Mazan, M., Sage, S., Sanchez, A., & Widmer, G. (2020). The fecal microbiota of healthy donor horses and geriatric recipients undergoing fecal microbial transplantation for the treatment of diarrhea. *PLoS One*, *15*(3), e0230148. https://doi.org/10.1371/journal.pone.0230148

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A., & Zenger, K. R. (2019). Methods for normalizing microbiome data: An ecological perspective. *Methods in Ecology and Evolution*, *10*(3), 389–400. https://doi.org/10.1111/2041-210X.13115

McMurdie, P. J., & Holmes, S. (2014). Waste not, want not: Why rarefying microbiome data is inadmissible. *PLoS Computational Biology*, *10*(4), e1003531. https://doi.org/10.1371/journal.pcbi.1003531

Metcalf, J. L., Song, S. J., Morton, J. T., Weiss, S., Seguin-Orlando, A., Joly, F., Feh, C., Taberlet, P., Coissac, E., Amir, A., Willerslev, E., Knight, R., McKenzie, V., & Orlando, L. (2017). Evaluating the impact of domestication and captivity on the horse gut microbiome. *Scientific Reports*, *7*(1), 15497. https://doi.org/10.1038/s41598-017-15375-9

Miller, A. K., Westlake, C. S., Cross, K. L., Leigh, B. A., & Bordenstein, S. R. (2021). The microbiome impacts host hybridization and speciation. *PLoS Biology*, *19*(10), e3001417. https://doi.org/10.1371/journal.pbio.3001417

Moeller, A. H., Caro-Quintero, A., Mjungu, D., Georgiev, A. V., Lonsdorf, E. V., Muller, M. N., Pusey, A. E., Peeters, M., Hahn, B. H., & Ochman, H. (2016). Cospeciation of gut microbiota with hominids. *Science*, *353*(6297), 380–382. https://doi.org/10.1126/science.aaf3951

Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., Gonzalez, A., Fontana, L., Henrissat, B., Knight, R., & Gordon, J. I. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, *332*(6032), 970–974. https://doi.org/10.1126/science.1198719

Mueller, T., O'Hara, R. B., Converse, S. J., Urbanek, R. P., & Fagan, W. F. (2013). Social learning of migratory performance. *Science*, *341*(6149), 999–1002. https://doi.org/10.1126/science.1237139

Neish, A. S. (2009). Microbes in gastrointestinal health and disease. *Gastroenterology*, *136*(1), 65–80. https://doi.org/10.1053/j.gastro.2008.10.080

Nielsen, D. P., Harrison, J. G., Byer, N. W., Faske, T. M., Parchman, T. L., Simison, W. B., & Matocq, M. D. (2023). The gut microbiome reflects ancestry despite dietary shifts across a hybrid zone. *Ecology Letters*, *26*(1), 63–75. https://doi.org/10.1111/ele.14135

Nishida, A. H., & Ochman, H. (2018). Rates of gut microbiome divergence in mammals. *Molecular Ecology*, *27*(8), 1884–1897. https://doi.org/10.1111/mec.14473

Noonan, M. J., Fleming, C. H., Tucker, M. A., Kays, R., Harrison, A.-L., Crofoot, M. C., Abrahms, B., Alberts, S. C., Ali, A. H., Altmann, J., Antunes, P. C., Attias, N., Belant, J. L., Beyer, D. E., Jr., Bidner, L. R., Blaum, N., Boone, R. B., Caillaud, D., de Paula, R. C., … Calabrese, J. M. (2020). Effects of body size on estimation of mammalian area requirements. *Conservation Biology*, *34*(4), 1017–1028. https://doi.org/10.1111/cobi.13495

Ogier, J.-C., Pagès, S., Galan, M., Barret, M., & Gaudriault, S. (2019). rpoB, a promising marker for analyzing the diversity of bacterial communities by amplicon sequencing. *BMC Microbiology*, *19*(1), 171. https://doi.org/10.1186/s12866-019-1546-z

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Wagner, H., & Oksanen, M. J. (2013). *Package 'vegan'* (2.9) [computer software].

Pansu, J., Hutchinson, M. C., Anderson, T. M., te Beest, M., Begg, C. M., Begg, K. S., Bonin, A., Chama, L., Chamaillé-Jammes, S., Coissac, E., Cromsigt, J. P. G. M., Demmel, M. Y., Donaldson, J. E., Guyton, J. A., Hansen, C. B., Imakando, C. I., Iqbal, A., Kalima, D. F., Kerley, G. I. H., … Pringle, R. M. (2022). The generality of cryptic dietary niche differences in diverse large-herbivore assemblages. *Proceedings of the National Academy of Sciences*, *119*(35), e2204400119. https://doi.org/10.1073/pnas.2204400119

Philippot, L., Andersson, S. G. E., Battin, T. J., Prosser, J. I., Schimel, J. P., Whitman, W. B., & Hallin, S. (2010). The ecological coherence of high bacterial taxonomic ranks. *Nature Reviews Microbiology*, *8*(7), 523–529. https://doi.org/10.1038/nrmicro2367

R Core Team. (2020). *R: A language and environment for statistical computing (3.6.1) [computer software]*. R Foundation for Statistical Computing.

Reed, K. J., Kunz, I. G. Z., Scare, J. A., Nielsen, M. K., Metcalf, J. L., Coleman, R. J., & Coleman, S. J. (2017). Can fecal samples be used to inform about microbial communities of the equine hindgut? *Journal of Equine Veterinary Science*, *52*, 52–53.

Regalado, J., Lundberg, D. S., Deusch, O., Kersten, S., Karasov, T., Poersch, K., Shirsekar, G., & Weigel, D. (2020). Combining whole-genome shotgun sequencing and rRNA gene amplicon analyses to improve detection of microbe–microbe interaction networks in plant leaves. *The ISME Journal*, *14*(8), 2116–2130. https://doi.org/10.1038/s41396-020-0665-8

Rosshart, S. P., Herz, J., Vassallo, B. G., Hunter, A., Wall, M. K., Badger, J. H., McCulloch, J. A., Anastasakis, D. G., Sarshad, A. A., Leonardi, I., Collins, N., Blatter, J. A., Han, S.-J., Tamoutounour, S., Potapova, S., St, F., Claire, M. B., Yuan, W., Sen, S. K., … Rehermann, B. (2019). Laboratory mice born to wild mice have natural microbiota and model human immune responses. *Science*, *365*(6452), eaaw4361. https://doi.org/10.1126/science.aaw4361

Rubenstein, D. I. (1986). Ecology and sociality in horses and zebras. In *Ecological aspects of social evolution* (pp. 282–302). Princeton University Press.

Russell, W. L. (1941). Inbred and hybrid animals and their value in research. In *Biology of the laboratory mouse* (pp. 325–348). Blakiston.

Sanders, J. G., Beichman, A. C., Roman, J., Scott, J. J., Emerson, D., McCarthy, J. J., & Girguis, P. R. (2015). Baleen whales host a unique gut microbiome with similarities to both carnivores and herbivores. *Nature Communications*, *6*(1), 8285. https://doi.org/10.1038/ncomms9285

Schieltz, J. M., & Rubenstein, D. I. (2015). Caught between two worlds: Genes and environment influence behaviour of plains×Grevy's zebra hybrids in central Kenya. *Animal Behaviour*, *106*, 17–26. https://doi.org/10.1016/j.anbehav.2015.04.026

Schloss, P. D. (2024). Rarefaction is currently the best approach to control for uneven sequencing effort in amplicon sequence analyses. *mSphere*, *9*, e00354-23. https://doi.org/10.1128/msphere.00354-23

Schluter, J., & Foster, K. R. (2012). The evolution of mutualism in gut microbiota via host epithelial selection. *PLoS Biology*, *10*(11), e1001424. https://doi.org/10.1371/journal.pbio.1001424

Shi, N., Li, N., Duan, X., & Niu, H. (2017). Interaction between the gut microbiome and mucosal immune system. *Military Medical Research*, *4*(1), 14. https://doi.org/10.1186/s40779-017-0122-9

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Sommer, F., & Bäckhed, F. (2013). The gut microbiota—Masters of host development and physiology. *Nature Reviews Microbiology*, *11*(4), 227–238. https://doi.org/10.1038/nrmicro2974

Song, S. J., Sanders, J. G., Delsuc, F., Metcalf, J., Amato, K., Taylor, M. W., Mazel, F., Lutz, H. L., Winker, K., Graves, G. R., Humphrey, G., Gilbert, J. A., Hackett, S. J., White, K. P., Skeen, H. R., Kurtis, S. M., Withrow, J., Braile, T., Miller, M., … Knight, R. (2020). Comparative analyses of vertebrate gut microbiomes reveal convergence between birds and bats. *MBio*, *11*(1), e02901-19. https://doi.org/10.1128/mBio.02901-19

Soria, C. D., Pacifici, M., Di Marco, M., Stephen, S. M., & Rondinini, C. (2021). COMBINE: A coalesced mammal database of intrinsic and extrinsic traits. *Ecology*, *102*(6), e03344. https://doi.org/10.1002/ecy.3344

Stothart, M. R., McLoughlin, P. D., & Poissant, J. (2023). Shallow shotgun sequencing of the microbiome recapitulates 16S amplicon results and provides functional insights. *Molecular Ecology Resources*, *23*(3), 549–564. https://doi.org/10.1111/1755-0998.13713

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G., Brochmann, C., & Willerslev, E. (2007). Power and limitations of the chloroplast trn L (UAA) intron for plant DNA barcoding. *Nucleic Acids Research*, *35*(3), e14. https://doi.org/10.1093/nar/gkl938

Tanaka, M., & Nakayama, J. (2017). Development of the gut microbiota in infancy and its impact on health in later life. *Allergology International*, *66*(4), 515–522. https://doi.org/10.1016/j.alit.2017.07.010

Tasnim, N., Abulizi, N., Pither, J., Hart, M. M., & Gibson, D. L. (2017). Linking the gut microbial ecosystem with the environment: Does gut health depend on where we live? *Frontiers in Microbiology*, *8*, 299664. https://doi.org/10.3389/fmicb.2017.01935

Trevelline, B. K., & Kohl, K. D. (2022). The gut microbiome influences host diet selection behavior. *Proceedings of the National Academy of Sciences*, *119*(17), e2117537119. https://doi.org/10.1073/pnas.2117537119

Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J. R., Vázquez-Baeza, Y., Birmingham, A., Hyde, E. R., & Knight, R. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*, *5*(1), 27. https://doi.org/10.1186/s40168-017-0237-y

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227. https://doi.org/10.1038/nature11053

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., & Glöckner, F. O. (2014). The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. *Nucleic Acids Research*, *42*(D1), D643–D648. https://doi.org/10.1093/nar/gkt1209

Zhang, C., Derrien, M., Levenez, F., Brazeilles, R., Ballal, S. A., Kim, J., Degivry, M.-C., Quéré, G., Garault, P., van Hylckama Vlieg, J. E. T., Garrett, W. S., Doré, J., & Veiga, P. (2016). Ecological robustness of the gut microbiota in response to ingestion of transient food-borne microbes. *The ISME Journal*, *10*(9), 2235–2245. https://doi.org/10.1038/ismej.2016.13

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Abraham, J. O., Lin, B., Miller, A. E., Henry, L. P., Demmel, M. Y., Warungu, R., Mwangi, M., Lobura, P. M., Pallares, L. F., Ayroles, J. F., Pringle, R. M., & Rubenstein, D. I. (2024). Determinants of microbiome composition: Insights from free-ranging hybrid zebras (*Equus quagga* × *grevyi*). *Molecular Ecology*, *33*, e17370. https://doi.org/10.1111/mec.17370

1365294x, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17370, Wiley Online Library on [28/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License