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Interactions between social groups of colobus monkeys (*Colobus vellerosus*) explain similarities in their gut microbiomes

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The gut microbiome is structured by social groups in a variety of host taxa. Whether this pattern is driven by relatedness, similar diets or shared social environments is under debate because few studies have had access to the data necessary to disentangle these factors in wild populations. We investigated whether diet, relatedness or the 1 m proximity network best explains differences in the gut microbiome among 45 female colobus monkeys in eight social groups residing at Boabeng-Fiema, Ghana. We combined demographic and behavioural data collected during May – August 2007 and October 2008 – April 2009 with 16S rRNA sequencing of faecal samples collected during the latter part of each observation period. Depending on the beta diversity index, social group identity explained 19–28% of the variation in gut microbiome beta diversity. When comparing the predictive power of dietary dissimilarity, relatedness and connectedness in the 1 m proximity network, the models with social connectedness received the strongest support, even in our analyses that excluded within-group dyads. This novel finding indicates that microbes may be transmitted during intergroup encounters, which could occur either indirectly via shared environments or directly via social contact. Lastly, some of the gut microbial taxa that appear to be transmitted via 1 m proximity are associated with digestion of plant material. Further research is needed to investigate whether this type of gut microbe transmission yields health benefits, which could provide an incentive for the formation and maintenance of social bonds within and between social groups.

The gut microbiome consists of thousands of species that affects its host's nutritional status, immune function and behaviour (McFall-Ngai et al., 2013). Gut microbiome composition is associated with parasite resistance and stress response of hosts in the wild (Koch & Schmid-Hempel, 2011; Vlčková et al., 2018) and with obesity in captive settings (Turnbaugh et al., 2006). Because of these potential health consequences, it is important to investigate the acquisition and maintenance of the gut microbiome (Amato, 2016; Archie & Tung, 2015), especially in wild vertebrate populations.

The gut microbiome of individuals or social groups become more distinct with geographical distance (Barelli et al., 2015; Grieneisen et al., 2019; Hansen et al., 2019; Hird, Carstens, Cardiff, Dittmann, & Brumfield, 2014; Lankau, Hong, & Mackie, 2012; Phillips et al., 2012), and the microbiome is structured by social group or family co-residency in a variety of host taxa, such as humans (Lax et al., 2014; Song et al., 2013; Yatsunenko et al., 2012), nonhuman primates (Amato et al., 2017; Degnan et al., 2012; Goodfellow et al., 2019; Orkin, Webb et al., 2019; Springer et al., 2017; Tung et al., 2015), carnivores (Leclaire, Nielsen, & Drea, 2014; Theis et al., 2013), birds (White et al., 2010) and insects (Anderson et al., 2012; Koch & Schmid-Hempel, 2011). Recent studies of nonhuman primates further highlight the importance of social groups in structuring gut microbiomes. The gut microbiome of immigrant male yellow baboons, *Papio cynocephalus*, converged over time with that of their new group members (Grieneisen, Livermore, Alberts, Tung, & Archie, 2017), and the gut microbiomes of white-thighed black-and-white colobus, *Colobus vellerosus*, diverged over the course of 9 months after a social group fissioned into two daughter groups (Goodfellow et al., 2019).

Within host populations, gut microbiomes diverge with increasing home range separation, potentially due to dietary

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differences, lower degrees of relatedness or lack of shared social environments (Archie & Tung, 2015; Björk, Dasari, Grieneisen, & Archie, 2019). Diet is suggested to be one of the most important factors affecting the gut microbiome (Voreades, Kozil, & Weir, 2014). Gut microbial composition fluctuates within hosts with seasonal or experimental dietary changes (Hicks et al., 2018; Mallott, Amato, Garber, & Malhi, 2018; Orkin, Campos et al., 2019). Dietary similarities, both within and between groups, may explain whether social groups have distinct gut microbiomes (Orkin, Webb et al., 2019). Alternatively, gut microbial similarity among group members could also reflect the genetic similarity of hosts when some closely related individuals remain together in their natal groups. Because the host genetic make-up can affect microbe colonization (Spor, Koren, & Ley, 2011; van Opstal & Bordenstein, 2015) and genomic regions are associated with gut microbial composition (Bonder et al., 2016; Leamy et al., 2014), gut microbial similarity is expected to increase with genetic relatedness.

It is therefore surprising that genetic differentiation between baboon populations was a poor predictor of their gut microbiome (Grieneisen et al., 2019), and that relatedness did not have a significant effect on the gut microbiome in some studies of humans (Rothschild et al., 2018), nonhuman primates (Grieneisen et al., 2017; Moeller et al., 2016) and carnivores (Leclaire et al., 2014). Moeller et al. (2016) suggested that this may be due to an overriding effect of transmission among unrelated social partners. Social transmission may occur indirectly via shared environments or directly via physical contact between hosts, however, these two transmission routes are hard to tease apart in observational studies where individuals in close physical contact also share environments (Archie & Tung, 2015). Lax et al. (2014) suggested that touching common surfaces may facilitate microbiome transmission within households (i.e. indirect social transmission). Direct social contact such as grooming or sitting in body contact further increases microbiome transmission (i.e. direct social transmission) between close social partners within social groups of monkeys (black howler monkey, *Alouatta pigra*: Amato et al., 2017; *P. cynocephalus*: Grieneisen et al., 2017; Tung et al., 2015) and lemurs (red-bellied lemur, *Eulemur rubriventer*: Raulo et al., 2017). Gut microbiomes are also more similar among socially connected than disconnected siblings and married couples (Dill-McFarland et al., 2019). Although social connectedness did not predict gut microbiome similarity between nongroup members in Verreaux's sifaka, *Propithecus verreauxi*, there may not be an association between intergroup interactions and gut microbiome similarity when groups are rarely in close physical proximity, as is the case for sifakas (Perofsky, Lewis, Abondano, Di Fiore, & Meyers, 2017). Taken together, these studies indicate that gut microbes are transmitted via social interactions within social groups, while the between-group social transmission of gut microbes has not yet been demonstrated in wild primates.

To investigate whether the pattern of increasing between-individual differences in the gut microbiome (i.e. beta diversity) with home range separation is best explained by lower dietary overlap, relatedness or social connectedness, we focus on the black-and-white colobus monkeys, *C. vellerosus*, at Boabeng-Fiema, Ghana. This is one of several rare species of arboreal leaf-eating monkeys distributed across the forested regions of the African tropics, and it is closely related to guerezas, *Colobus guereza*, and western black-and-white colobus, *Colobus polykomos* (Ting, 2008). At Boabeng-Fiema, all colobus social groups utilize a highly folivorous diet, but the most important food species differ between social groups (Saj & Sicotte, 2007; Teichroeb & Sicotte, 2009). More seeds and fruits are available during the dry season, during which they eat up to 43% of these food items (Teichroeb & Sicotte, 2017). To break down hard-to-digest items in their primarily folivorous diet (Saj & Sicotte, 2007; Teichroeb & Sicotte, 2009), they rely on behavioural traits, physiological traits and their gut microbiome (Amato et al., 2016; Lambert, 1998). Possibly due to constraints imposed by their highly folivorous diet, colobus monkeys spend a low percentage of their time engaging in direct social activities such as grooming (Teichroeb, Saj, Paterson, & Sicotte, 2003). Female colobus spend on average 3% of their time within 1 m and 0.1% of their time grooming each female group member (Wikberg, Ting, & Sicotte, 2014b). However, females still form preferred friendships, which are only occasionally based on kinship and never based on their relatively weakly expressed dominance hierarchies (Wikberg et al., 2013, 2014b, 2015, 2014a). Instead, females prefer to affiliate with females with similar immigration status (Wikberg, Ting, & Sicotte, 2014a; 2014b) in this population where all males and half of the females disperse (Sicotte et al., 2017; Teichroeb, Wikberg, & Sicotte, 2011, 2009; Wikberg, Sicotte, Campos, & Ting, 2012). This flexible female dispersal pattern results in social groups with different female kin composition and some close maternal female kin residing in different social groups (Wikberg et al., 2012). Neighbouring social groups encounter each other in the large zones of home range overlap on an almost daily basis. During these encounters, social groups sometimes chase each other away from food trees, while at other times, they engage in affiliative or sexual between-group interactions (Sicotte & MacIntosh, 2004; Teichroeb & Sicotte, 2017).

The frequent between-group interactions coupled with variation in diet and relatedness within and between social groups makes this a good study population to investigate whether the pattern of increasing gut microbial beta diversity with home range separation is best explained by lower degrees of dietary similarity, relatedness or social connectedness. We take a cross-sectional approach using observational and genetic data from eight social groups to first test whether the gut microbiome was structured by social groups. We then evaluated which factors explained gut microbiome beta diversity between females across different social groups. Although beta diversity within groups was best predicted by the group's 1 m proximity network (Wikberg et al., n.d.), there are greater differences in diet and relatedness between groups than within groups. Therefore, we expected gut microbiome beta diversity not only to increase with distance in the 1 m proximity network but also to decrease with dietary similarity and relatedness. Finally, the significant predictor from the analyses above (social connectedness) was used in a subsequent population-level analysis of operational taxonomic unit (OTU) abundance to determine which microbial taxa may be socially transmitted. Our definition of social transmission includes both direct social transmission via physical contact and indirect social transmission via shared substrates (e.g. Perofsky et al., 2017), and we will not attempt here to tease apart these two social transmission routes. Males and females of all age classes were used to create social networks, but the gut microbiome data are only available for adult females. Therefore, our analyses of beta diversity focus on adult females.

METHODS

Behavioural Data Collection

Demographic data have been collected since 2000 from the black-and-white colobus monkeys (*C. vellerosus*) at Boabeng-Fiema, Ghana. In this study, we also use behavioural and ecological data as well as DNA samples from eight social groups (Appendix, Fig. A1) collected during two study periods: the rainy season May–August 2007 and the pre-dry and dry seasons October 2008–April 2009

(Appendix, Table A1). During this period, the study groups contained 3–9 adult (i.e. parous) females (Appendix, Table A1), 1–4 adult males and 8–24 immatures. Our research adheres to ASAB/ABS Guidelines for the Use of Animals in Research and the laws of Ghana, and data collection was approved by the Boabeng-Fiema Monkey Sanctuary's management committee, Ghana Wildlife Division and the University of Calgary's Animal Care Committee (BI 2006-28, BI 2009-25).

We recorded each social group's location every hour using a map with trails, roads, villages and large trees (>40 cm diameter at breast height, DBH) in order to determine home ranges (Appendix, Fig. A1). During 10 min focal samples (Altmann, 1974) of adult females, we continuously recorded all social behaviours (including the identity of the interactant and the duration of the behaviour) and plant species and part (i.e. mature leaf, young leaf, flower, fruit, seed or other) for each ingested food item. Females fed on a total of 210 food item–plant species combinations, and to assess dietary differences, we calculated Sørensen dissimilarity indices using ingested plant parts and plant species during focal samples. We choose this diversity index because it only takes the presence or absence of an ingested food item into account, which we have a robust estimate of using the focal data. The Sørensen dissimilarity indices in our data set had a high median value of 0.83 and were lower within than between social groups (Appendix, Fig. A2).

During the first and second data collection period, we observed 61 and 285 between-group encounters (i.e. two social groups located within 50 m of each other), respectively. Of these encounters, 53% lacked female aggression and 35% lacked male aggression. Because close proximity between individuals of different social groups are rare and unlikely to be recorded during focal sampling, we recorded approaches to 1 m ad libitum (Altmann, 1974). Some of these approaches only led to brief close proximity while others led to prolonged contact like copulations, grooming and play. We created an undirected proximity network based on the presence and absence of approaches to 1 m between all individuals ($N = 177$ adult females, adult males and immatures) present in the eight study groups. We used the software UCINET (Borgatti, Everett, & Freeman, 2002) to compute inverse shortest path length (i.e. geodesic distance) in the 1 m proximity network (hereafter referred to as social connectedness): 1/(the number of steps (i.e. recorded interaction ties) in the shortest path from one individual to another). Social group members were in 1 m proximity with each other (i.e. an inverse path length of 1) or separated by two to three partners (i.e. an inverse path length of 0.5 and 0.33) (Appendix, Fig. A2). The inverse path length for males and females belonging to different social groups ranged from 0 to 1 (see Results, Fig. 1, Appendix, Fig. A2). The seemingly unconnected individuals in the 2007 data set were most likely unconnected because we only had access to data collected from a 3-month period. These individuals were connected and separated with up to eight steps in the 2008–2009 network, which was based on 6 months of data.

Genetic Data Collection

We collected faecal samples during June–August 2007 and January–April 2009. Immediately after a female defecated, we collected approximately 1 g of faeces and dissolved it in 6 ml of RNAlater. The samples were stored in a refrigerator at the field site until the end of the field season when they were transported to the Ting laboratory (University of Oregon, Eugene, OR, U.S.A.) and stored in a -20 °C freezer. Note that we lack information on soil type, which was driving between-site differences in the gut microbiomes in a large-scale study of terrestrial baboons (Grieneisen et al., 2019). However, our samples were collected from arboreal primates within a small study area, and sampling site does not have a significant effect on beta diversity in our study population (Goodfellow et al., 2019).

We extracted DNA from the samples and genotyped the extracts at 17 short tandem repeat loci (STR) as previously described (Wikberg et al., 2012). To make sure that the samples used in the relatedness and gut microbiome analyses were collected from the correct individual, we compared the STR genotypes obtained from these samples with a second sample collected from the same individual at a different time. We calculated dyadic estimated relatedness values (R) in MLRelate (Kalinowski, Wagner, & Taper, 2006) because this method provided the most accurate relatedness estimates in our study population (Wikberg et al., 2012). We used R values calculated from STR loci rather than theoretical relatedness (r) calculated from pedigrees, because R values predict kinship relatively accurately in our study population (Wikberg et al., 2014a) and they are more accurate than r in studies such as ours with limited access to pedigrees (Forstmeier, Schielzeth, Mueller, Ellegren, & Kempenaers, 2012; Robinson, Simmons, & Kennington, 2013). The median female relatedness was low both within and between social groups, but there were at least some closely related females residing in the same social groups (Appendix, Fig. A2).

For generating the gut microbial data, we conducted fresh DNA extracts from 61 previously genotyped samples from 45 females (Appendix, Table A1) using the QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA, U.S.A.) with a modified protocol. More details regarding the extraction protocol are presented in the Appendix and in Goodfellow et al. (2019). The V4 hypervariable region of the bacterial 16S ribosomal RNA gene was amplified and libraries were prepared using the 515F and 806R primers containing 5′ Illumina adapter tails and dual indexing barcodes, and libraries were sequenced as part of a 150 bp paired-end sequencing run on the Illumina NextSeq platform following Goodfellow et al. (2019). We obtained a mean read depth of 127 628 per sample (range 86 924–166 438). Then, we used a custom pipeline (https://github.com/kstagaman/Process_16S) for quality filtering and assembly (see Appendix). We performed de novo OTU picking in UCLUST (Edgar, 2010), and sequences with 97% overlap were defined as belonging to the same bacterial operational taxonomic unit (OTU). After this processing, we had a total of 2597 OTUs and an average of 89 483 reads per sample (range 59 817–120 119). To further guard against sequencing errors, we filtered out OTUs with a frequency lower than 0.00005 as recommended (Bokulich et al., 2012). After filtering, the 2007 data set contained 450 OTUs and the 2009 data set contained 396 OTUs. The mean read depth was 88 346 (range 59 005–118 633). We did not rarefy the data set to an even read depth, because it is recommended against (McMurdie & Holmes, 2014). First, rarefying leads to increased false positives and decreased true positives, especially in data sets with read depths comparable to ours (Pereira, Wallroth, Jonsson, & Kristiansson, 2018). Second, unrarefied counts are particularly accurate when using our measure of beta diversity – weighted UniFrac distances (McMurdie & Holmes, 2014).

We initially calculated four different measures of gut microbiome beta diversity (Sørensen dissimilarity index, Bray–Curtis dissimilarity index, unweighted UniFrac distances and weighted UniFrac distances) in the R package 'vegan' (Oksanen et al., 2017). Because the two presence/absence indices were strongly correlated with each other (Sorenson dissimilarity indices and unweighted UniFrac distances: Mantel $r = 0.93$, $P = 0.001$) as were the two abundance indices (Bray–Curtis dissimilarity indices and weighted UniFrac distances: Mantel $r = 0.77$, $P = 0.001$), in our analyses, we only used the one presence/absence index (unweighted UniFrac distances) and the one abundance index (weighted UniFrac distances) that take phylogenetic relationships of OTUs into account.

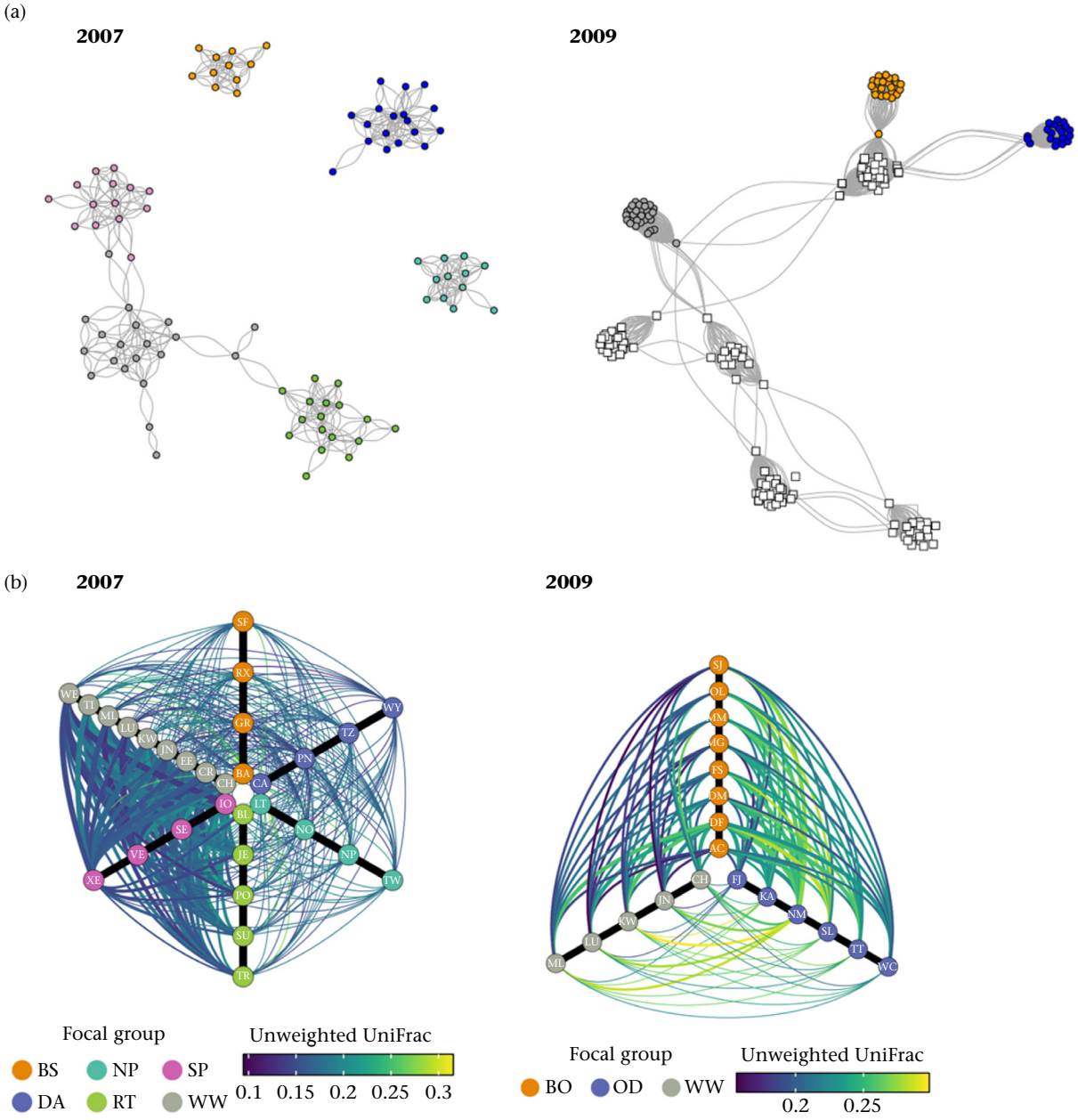

Figure 1. (a) Social networks for the entire population in 2007 and 2009, where each group member is depicted as a node (circles in different colours/white squares represent individuals in the different groups that were used/not used for the analyses of behavioural predictors of gut microbiome similarity; see key to symbols) and individuals observed in 1 m proximity are connected with lines. (b) Social networks for females included in the analyses of behavioural predictors of gut microbiome similarity in 2007 and 2009, with lines connecting between-group dyads (i.e. nodes of different colour) and where line colour represents gut microbiome beta diversity (i.e. unweighted UniFrac distances), ranging from similar (dark) to dissimilar (light), and thickness indicates social connectedness, ranging from strongly connected (thick) to more disconnected (thin). The black lines connect group members and are not weighted based on beta diversity or social connectedness.

Data Analyses

We combined the 2007 and 2009 data sets and included study year (i.e. season) and individual identity (ID) as predictor variables whenever possible (i.e. permutational multivariate analysis of variance and the generalized linear mixed models) while we had to create squared interaction matrices for each study year separately when using matrix correlations (i.e. Mantel tests and Moran's test for autospatial correlations). We only used the full data set ($N = 61$ samples from 45 females) for the initial analysis regarding the effect of social group identity. All subsequent analyses examined the effects of behavioural variables on beta diversity in a subset ($N = 49$ samples from 42 unique females) from which we removed (1) the second sample collected from same female in the same year, (2) one adult female with incomplete dietary information and (3) social groups from which the majority of females remained unsampled to make sure we had a representative sample of social connectedness from each social group.

The initial analysis investigated the effects of season, social group, individual identity and read depth on beta diversity of all dyads in the full data set ($N = 61$ samples from 45 females in 2007 and 2009) using permutational multivariate analysis of variance

(PERMANOVA) with 10 000 permutations using the 'adonis' function in the R package 'vegan' (Oksanen et al., 2017). The terms were added sequentially in the order listed above.

We used nonparametric Mantel correlations implemented in the R package 'vegan' (Oksanen et al., 2017) to investigate whether the two measures of gut microbiome beta diversity were correlated with home range separation (0 = same social group and home range; 1 = different social groups but adjacent home ranges; 2 = different social groups and nonadjacent home ranges) using beta diversity indices from 30 samples from unique females in six social groups in 2007 and 19 samples from unique females in three social groups in 2009. We used beta diversity indices of all dyads, but analysed the two years separately.

To investigate which combination of dyadic traits predicted gut microbiome beta diversity between females, we created generalized linear mixed models (GLMMs) with the outcome variable gut microbiome beta diversity using the 'beta family' function in the package 'glmmTMB' (version 0.2.3; Brooks et al., 2017) in R (R Core Team, 2018). Again, we used 30 samples from unique females in six social groups in 2007 and 19 samples from unique females in three social groups in 2009. We created a null model that did not contain any fixed effects, alternative models with one fixed effect that represented one of the hypotheses outlined in the Introduction (dietary dissimilarities, *R* values or social connectedness) and a full model with all three predictor variables. We included data collection year as a fixed effect in all alternative models because the two sampling years occurred in different seasons and several other studies showed strong seasonal shifts in gut microbiome composition (Amato et al., 2015; Hicks et al., 2018; Orkin, Campos et al., 2019; Smits et al., 2017; Springer et al., 2017). All numerical predictor variables were centred and scaled (Schielzeth, 2010). We included social group and focal identities as random effects in all GLMMs, including the null models. We did not have any issues with collinearity based on low variance inflation factors (VIF) for the full models (all VIF < 1.43). We evaluated the support for each model using Akaike's information criterion (AIC) (Akaike, 1974), and this approach allowed us to determine which hypotheses (diet, relatedness or social connectedness) was best supported by our data (Burnham & Anderson, 2002). In these analyses, two models received similar support (with a difference in AIC < 4). Therefore, we took model selection uncertainly into account by averaging coefficients across these two most well-supported models (Burnham & Anderson, 2002) using the R package 'MUMIN' (Barton, 2013). We present these averaged coefficients in the Results, and when their 95% confidence intervals did not overlap zero, we conclude that the fixed effect predicted beta diversity. We also include the output for each of the well-supported models in the Appendix (Table A2). In the first set of analyses, we included dyads that resided in the same social group and dyads that resided in different social groups. To make sure that the effect of social connectedness was not driven by the close social bonds within social groups, we repeated the analyses with between-group dyads only.

To infer which of the gut microbial taxa may be transmitted via close proximity, which was a better predictor of beta diversity than diet and relatedness (see Results), we investigated whether the abundance of each OTU was correlated with geodesic distance in the 1 m approach network using Moran's test for autospatial correlations implemented in the package 'ape' (Paradis, Claude, & Strimmer, 2004). We included within-group and between-group dyads in this analysis ($N = 342$ dyads). We counted the number of OTUs in each phylum (or family) that were socially structured based on the autospatial correlation results. We conducted hypergeometric tests to investigate whether this number was higher than expected by chance based on the total number of OTUs in the phylum (or family) using the 'phyper' function implemented in R. In all analyses of taxonomic differences, we used the 10% false discovery rate (FDR) to correct *P* values for multiple testing (sensu Tung et al., 2015). The gut microbial taxa we expected to be shaped by sociality are listed in the Appendix, Table A3 (Amato et al., 2017; Goodfellow et al., 2019; Tung et al., 2015).

RESULTS

Factors Predicting Gut Microbiome Beta Diversity

We investigated the relative effects of year, social group, individual ID and read depth in the full data set (PERMANOVA: $N = 61$ samples collected during 2007–2009). Of the observed variation in the taxonomic composition of the gut microbiome (i.e. beta diversity), individual identity explained the largest percentage (54–55% depending on which beta diversity index was used as outcome variable), social group identity explained a more moderate percentage (19–28%), while year explained much smaller percentage (8–12%) (Table 1). Read depth did not have a significant effect on beta diversity (Table 1).

Gut microbiome beta diversity and home range separation were correlated in the 2007 data set ($N = 870$ dyads in 6 social groups, Mantel tests: unweighted UniFrac distance: $r = 0.22$, $P = 0.002$; weighted UniFrac distance: $r = 0.10$, $P = 0.049$) and in the 2009 data set ($N = 342$ dyads in 3 social groups, Mantel tests: unweighted UniFrac distance: $r = 0.36$, $P = 0.005$; weighted UniFrac distance: $r = 0.20$, $P = 0.024$), meaning that females residing farther from each other had less similar gut microbiomes. This pattern can potentially be explained by group members having more similar diets, higher relatedness or stronger social connectedness than nongroup members (Appendix, Fig. A2).

We created several competing generalized linear mixed models to investigate which of the three hypotheses best explained increasing beta diversity with home range separation: dietary dissimilarity, relatedness or social connectedness, controlling for data collection year. In our data set with both within-group and between-group dyads ($N = 1212$ dyads in 2007–2009), the full models and the models with social connectedness received the greatest support (Table 2). Gut microbiome beta diversity was predicted by year (unweighted UniFrac coefficient estimate = 0.316, 95% CI: 0.274, 0.357; weighted UniFrac coefficient estimate = 0.202, 95% CI: 0.154, 0.249), and females had more similar gut microbiomes during the rainy season of 2007 than during the dry season of 2009 (Fig. 2). Gut microbiome beta diversity was also predicted by social connectedness (unweighted UniFrac coefficient estimate = -0.085, 95% CI: -0.098, -0.072; weighted UniFrac coefficient estimate = -0.044, 95% CI: -0.062, -0.026), and females located further apart in the social network had less similar gut microbiomes (Figs. 1, 2). In contrast, gut microbiome beta diversity was not predicted by diet (unweighted UniFrac coefficient estimate = 0.009, 95% CI: -0.004, 0.021; weighted UniFrac coefficient estimate = -0.016, 95% CI: -0.032, 0.001) or relatedness (unweighted UniFrac coefficient estimate = 0.002, 95% CI: -0.008, 0.013; weighted UniFrac coefficient estimate = 0.001, 95% CI: -0.013, 0.015).

To assess whether the effect of social connectedness on gut microbiome beta diversity was driven by closely connected within-group dyads having very similar gut microbiomes, we repeated the analyses with between-group dyads only ($N = 966$ dyads). The full models and the social connectedness models were again the strongest supported models (Table 2). Beta diversity was predicted by year (unweighted UniFrac coefficient estimate = 0.336, 95% CI: 0.291, 0.381; weighted UniFrac coefficient estimate = 0.232, 95% CI: 0.174, 0.291) and social connectedness (unweighted UniFrac

Table 1
Results from the PERMANOVA with factors added sequentially in the order listed in the table

Beta diversity index	Factor	*df*	Sums of squares	Mean squares	*F*	R^2	*P*
Unweighted UniFrac	Year	1	0.103	0.103	12.325	0.084	<0.001
	Group	7	0.347	0.050	5.943	0.282	<0.001
	ID	39	0.663	0.017	2.035	0.538	<0.001
	Read depth	1	0.010	0.010	1.185	0.008	0.249
Weighted UniFrac	Year	1	0.136	0.136	12.277	0.118	<0.001
	Group	7	0.219	0.031	2.812	0.189	<0.001
	ID	39	0.639	0.016	1.477	0.553	<0.001
	Read depth	1	0.018	0.018	1.637	0.016	0.104

Table 2
The competing GLMMs' fixed effects, Akaike's information criterion (AIC), delta (i.e. difference in AIC between the current model and the best-fit model) and Akaike weights (i.e. relative likelihood of the model) when including within-group and between-group dyads and only between-group dyads

Outcome variable	Fixed effect	AIC	Delta	Weight
Within-group and between-group dyads				
Unweighted UniFrac	Year + Social connectedness	-5247.30	0.00	0.73
	Year + Social connectedness + Diet + Relatedness	-5245.28	2.02	0.27
	Year + Diet	-5103.63	143.67	0.00
	Year + Relatedness	-5060.36	186.94	0.00
	_	-4940.73	306.58	0.00
Weighted UniFrac	Year + Social connectedness	-4684.20	0.00	0.56
	Year + Social connectedness + Diet + Relatedness	-4683.69	0.51	0.44
	Year + Relatedness	-4658.94	25.25	0.00
	Year + Diet	-4657.85	26.35	0.00
	_	-4614.36	69.84	0.00
Between-group dyads				
Unweighted UniFrac	Year + Social connectedness	-4307.62	0.00	0.76
	Year + Social connectedness + Diet + Relatedness	-4303.92	3.71	0.12
	Year + Diet	-4302.67	4.95	0.06
	Year + Relatedness	-4302.44	5.19	0.06
	_	-4119.55	188.07	0.00
Weighted UniFrac	Year + Social connectedness	-3770.21	0.00	0.64
	Year + Social connectedness + Diet + Relatedness	-3768.91	1.30	0.33
	Year + Diet	-3762.64	7.58	0.01
	Year + Relatedness	-3761.96	8.25	0.01
	_	-3713.73	56.48	0.00

coefficient estimate = -0.046, 95% CI: -0.086, -0.006; weighted UniFrac coefficient estimate = -0.086, 95% CI: -0.141, -0.031), but not by diet (unweighted UniFrac coefficient estimate = 0.004, 95% CI: -0.009, 0.017; weighted UniFrac coefficient estimate = -0.012, 95% CI: -0.031, 0.006) or relatedness (unweighted UniFrac coefficient estimate = -0.001, 95% CI: -0.013, 0.012; weighted UniFrac coefficient estimate = -0.009, 95% CI: -0.027, 0.009).

Socially Structured OTUs

Our data set contained OTUs from 14 phyla, of which the most well represented was Firmicutes, followed by Bacteroidetes, Spirochetes and Verrucomicrobia (Supplementary Material Fig. S1). In each social group, at least 70% of the OTUs belonged to the phylum Firmicutes (Supplementary Material Fig. S1) and at least 50% of the OTUs belonged to the families Lachnospiraceae and Ruminococcaceae in the phylum Firmicutes (Supplementary Material Fig. S2).

Social connectedness predicted differences in abundances for 73 of the 396 OTUs in the 2009 data set (Moran's *I* range: -0.27, -0.14, all $P < 0.05$; Supplementary Material Table S1). The number of OTUs with a significant relationship to social connectedness was greater than expected in the phylum Firmicutes (hypergeometric test: $N = 64$, $P < 0.001$). The numbers of socially structured OTUs in the phyla Bacteroidetes ($N = 6$), Planctomycetes ($N = 1$), Proteobacteria ($N = 1$) and Tenericutes ($N = 1$) were not greater than expected based on the total number of OTUs in these phyla (hypergeometric tests: all $P > 0.050$). The other phyla did not contain any socially structured OTUs. Four families had a higher than expected number of socially structured OTUs: Bacteroidaceae ($N = 4$), Lachnospiraceae ($N = 20$), Peptococcaceae 2 ($N = 1$) and Ruminococcaceae ($N = 31$) (hypergeometric tests: all $P < 0.001$). There was also a greater than expected number of socially structured OTUs in 14 of 34 genera (Fig. 3).

Social connectedness predicted differences in abundances for one of the 450 OTUs in the 2007 data set (Moran's *I* range: -0.27, -0.14, all $P < 0.05$), which belonged to the phylum Firmicutes, the family Lachnospiraceae and the genus *Roseburia*. As a result, these taxa had a greater than expected number of socially structured OTUs (hypergeometric tests: all $P > 0.001$).

DISCUSSION

The aim of this study was to investigate whether the increase in gut microbiome beta diversity with home range separation in female colobus monkeys was best explained by diet, relatedness or sociality. Distance in the proximity network was a better predictor than diet and relatedness, similar to findings in more social primates (Amato et al., 2017; Perofsky et al., 2017; Raulo et al., 2017; Tung et al., 2015). Although these previous studies suggested that strong social bonds within social groups drive between-group differences in the gut microbiome after ruling out the effects of relatedness and diet, this is the first report of a relationship between gut microbiome beta diversity and social connectedness between individuals in different social groups. In contrast, gut

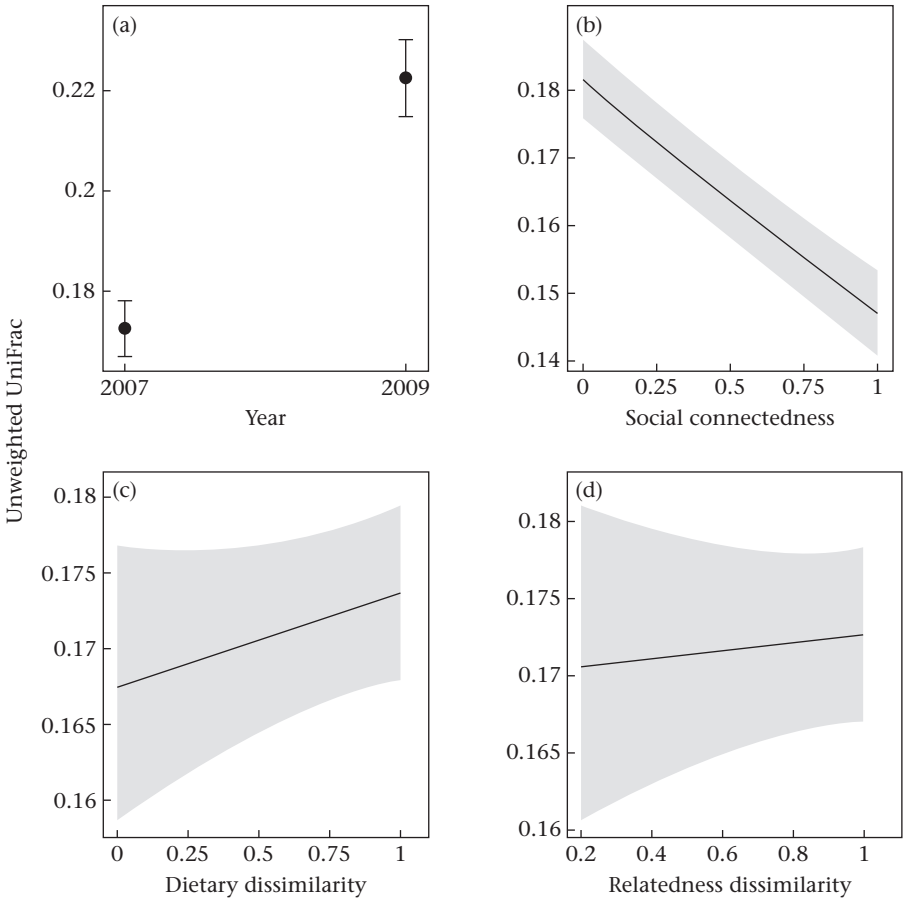

Figure 2. The relationship between gut microbiome beta diversity (unweighted UniFrac distances) and each fixed effect: (a) study year, (b) social connectedness, (c) dietary dissimilarity and (d) relatedness dissimilarity (1−*R* value). The lines show the relationships predicted by the GLMMs in the data set with within-group and between-group dyads, and the shaded areas represent the 95% confidence intervals. The original values for the fixed effects are shown here, but models reported in the text used fixed effects scaled to a mean of 0 and a standard deviation of 1.

microbiome dissimilarity between individuals residing in different social groups did not increase with grooming network distance in sifakas (Perofsky et al., 2017). These contrasting results may be due to the nature or frequency of the host population's between-group interactions. Sifakas rarely engage in direct interactions across groups even though groups show extensive home range overlap (Perofsky et al., 2017). Colobus monkeys sometimes engage in affiliative, sexual and playful behaviours with nongroup members (Sicotte & MacIntosh, 2004; Teichroeb et al., 2005, 2011), which differ from the almost exclusively aggressive nature of between-group encounters in some other taxa. Similar to these colobus monkeys, mountain gorillas, *Gorilla beringei beringei*, occasionally affiliate with members from other social groups (Forcina et al., 2019) and human foraging societies form extended social networks to optimize resource flow (Hamilton, Milne, Walker, Burger, & Brown, 2007). These extended networks could possibly affect their gut microbiome in similar ways as documented here in colobus monkeys.

To determine the consequences of such socially mediated transmission, the first step is to determine which types of microbes are transmitted this way. The OTUs associated with social transmission in this study included all taxa (family Porphyromonadaceae and genera *Parabacteroides* and *Coprococcus*) that diverged after a social group fission at our site (Goodfellow et al., 2019) and genera (*Bacteroides, Clostridium* and *Roseburia*) that were transmitted via grooming and close proximity in howlers (Amato et al., 2017). The close match in socially transmitted taxa in howlers and colobus is not particularly surprising given both have a folivorous diet and low degree of terrestriality, which are factors that influence the gut microbiome (Perofsky, Lewis, & Meyers, 2019). In contrast, the socially transmitted OTUs in our study did not overlap with those transmitted via grooming within social groups of baboons (Tung et al., 2015). This is surprising given the host species relatively close phylogenetic relationship, but baboons have a higher degree of terrestriality (Grieneisen et al., 2019), and the baboon groups incorporate a much higher percentage of grass corms and grass seeds in their diet (Tung et al., 2015) than our study groups do. Recent findings show that host phylogeny has a stronger effect than diet on gut microbiome composition (Amato et al., 2019), and it is thus possible that while phylogeny has the strongest overall effect on the gut microbiome, the same gut microbial taxa are structured by sociality in primates with similar lifestyles.

We found that the majority of socially transmitted OTUs belonged to the most dominant families in our host population and other folivorous primates (Barelli et al., 2015; Perofsky et al., 2017), the families Lachnospiraceae and Ruminococcaceae in the phylum Firmicutes. These taxa are well suited for breaking down hard-to-digest plant material (Biddle, Stewart, Blanchard, & Leschine, 2013), and it is therefore possible that socially transmitted gut microbes benefit hosts in terms of improved digestion of mature leaves, which make up the majority of the colobus diet (Saj & Sicotte, 2007). Several studies imply that socially mediated transmission benefits the host (Koch & Schmid-Hempel, 2011; Perofsky et al., 2017; Tung et al., 2015). For example, an experimental study of

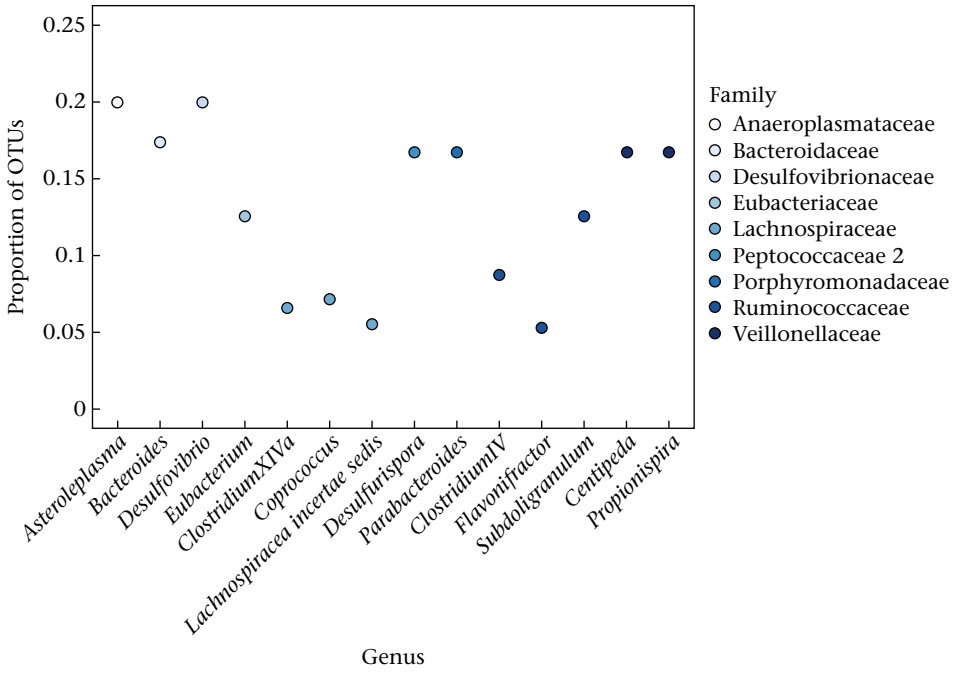

Figure 3. The proportion of operational taxonomic units (OTUs) whose abundance was correlated with connectedness in the proximity network for genera that contained a higher than expected number of socially structured OTUs.

bumble bees (*Bombus terrestris*) indicate that socially transmitted microbes protect the hosts against parasite infections (Koch & Schmid-Hempel, 2011). Our results support the notion that social transmission of gut microbes that benefit the host may occur in a wide range of gregarious species, including those with relatively low frequencies of social interactions. If social transmission sustains a healthy gut microbiome (as documented in Koch & Schmid-Hempel, 2011), it could provide an incentive for the formation and maintenance of social bonds within social groups (Lombardo, 2008; Münger, Montiel-Castro, Langhans, & Pacheco-López, 2018). Our findings leave open the as-of-yet unexplored possibility that social transmission of microbes may even explain the occurrence of friendly between-group encounters, especially in the absence of limiting resources such as fertile females and important food sources.

The results of this paper ultimately lead us to an important outstanding question, which is how gut microbes are transmitted among animals that spend considerably less time grooming or in direct contact than other primates with socially mediated gut microbe transmission (Amato et al., 2017; Raulo et al., 2017; Tung et al., 2015). It might be that microbes are transmitted directly during the occasions we observed nongroup members copulating, grooming and playing. However, it could also be that the microbes are transmitted indirectly between hosts when they are touching shared surfaces within a certain period (Münger et al., 2018). This reasoning is consistent with spatial proximity predicting the gut microbiome in other gregarious species with low frequencies of social behaviours like the Welsh Mountain ponies, *Equus ferus caballus* (Antwis, Lea, Unwin, & Shultz, 2018) and in more solitary species such as North American red squirrels, *Tamiasciurus hudsonicus* (Ren et al., 2017) and gopher tortoises (*Gopherus polyphemus*) (Yuan et al., 2015). The occurrences of direct and indirect social transmission are difficult to tease apart when the two are correlated and when brief physical contact between extragroup members often go unnoticed, but carefully designed studies in the future may be able to address this question.

Finally, relatedness and dietary differences within a season were not good predictors of beta diversity in comparison to social connectedness. In contrast, seasonal changes in diet may be associated with changes in the colobus gut microbiome, because beta diversity was higher during the 2009 dry season when their diet was more diverse than during the 2007 rainy season when they ate mostly mature leaves. We will continue to investigate whether this seasonal dietary switch is linked to changes in the gut microbiome, as previously reported from other species inhabiting seasonal environments (Amato et al., 2015; Hicks et al., 2018; Orkin, Campos et al., 2019; Smits et al., 2017; Springer et al., 2017). These authors concluded that gut microbiome dynamics determine nutrient uptake and are key for dietary flexibility (Amato et al., 2015; Hicks et al., 2018; Orkin, Campos et al., 2019; Smits et al., 2017; Springer et al., 2017), while the potential three-way interaction between social, dietary and gut microbial dynamics is still poorly understood. An interesting venue for further research is therefore to investigate whether the gut microbiomes of socially well-connected individuals map more quickly onto ecological changes, which could help them adjust to the rapidly changing environments that many wild animals inhabit today.

Data Availability

All raw data are stored in the PaceLab database hosted by the University of Calgary. The 16S sequencing data are available from NCBI's Short Read Archive (https://nam03.safelinks.protection.outlook.com/?url=http%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fbioproject%2F612541&data=02%7C01%7Ceva.wikberg%40utsa.edu%7Cb6d5395ca95847c55ff208d7c79b1315%7C3a228dfbc64744cb88357b20617fc906%7C0%7C0%7C637197341169638792&sdata=1fivclVV8oRG7ZuW0OoxM%2F27r%2BXCdlUiP83SaZc0vAY%3D&reserved=0). The data used for the analyses presented are available on Mendeley (https://data.mendeley.com/datasets/gkthnf3gyg/1).

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Supplementary Material

Supplementary material associated with this article is available, in the online version, at https://doi.org/10.1016/j.anbehav.2020.02.011.

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Appendix

Microbial Taxa Predicted To Be Structured by Social Connectedness

The last aim of this study was to investigate whether social connectedness was correlated with operational taxonomic unit (OTU) abundances in certain taxa (Table A3) previously reported as structured by social relationships (Amato et al., 2017; Tung et al., 2015). We also expected social connectedness to be correlated with the abundances of three gut microbial taxa that diverged between two daughter groups after a group fission (DA and NP in Fig. A1), because we suspected that this pattern was driven by social network changes (Goodfellow et al., 2019).

DNA Extraction, Amplification and Sequencing Protocols for the Gut Microbiome Analysis

We extracted DNA from 200 µl of sample using QIAmp DNA stool extraction protocol with the following modifications. In step 2, we added 50 µl of Proteinase K with overnight lysis before proceeding to step 3. In step 4, we pipetted all of the supernatant. In step 5, we used half of the InhibitEX tablet. In step 6, we centrifuged for 5 min. In step 9, we added 4 µl of RNAse and vortexed the sample for 15 s. In step 19, we used 50 µl of buffer AE and incubated it at 10 min. In step 20, we pipetted the same 50 µl of buffer AE back onto filter and incubated it at room temperature for 15 min. We centrifuged the sample at full speed for 2 min. Our DNA extraction protocol did not include a bead-beating step, which could bias against lysis-resistant taxa such as Gram-positive and spore-forming bacteria that are less likely to be dependent on direct social contact for transmission between hosts because they can survive for prolonged periods outside the host (Pollock, Glendinning, Wisedchanwet, & Watson, 2018; Yuan, Cohen, Ravel, Abdo, & Forney, 2012).

We determined the concentration of the extracts using Qubit dsDNA BR Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, U.S.A.) and diluted products to 2 nM for downstream reactions. We amplified the bacterial v4 region of the 16S ribosomal RNA gene using the following 515F and 806R primers containing 5′ Illumina adapter tails and dual indexing barcodes:

515F 5′ AATGATACGGCGACCACCGAGATCTACACTAGATCGCT ATGGTAATTGTGTGCCAGCMGCCGCGGTAA.

806R 5′ CAAGCAGAAGACGGCATACGAGATTCACCTAGAGTCAGT CAGCCGGACTACHVGGGTWTCTAAT.

We set the polymerase chain reactions (PCRs) with 12.5 µl of NEB Q5 Hot start 2× Master mix, 1.25 µl of 10 µM Primer mix, 1 µl of template DNA and 10.25 µl of MoBio certified DNA free water and used the following cycling protocol: 98 °C for 30 s (1×) followed by 98 °C for 10 s, 61 °C for 20 s and 72 °C for 20 s (20×), followed by 72 °C for 2 min and 4 °C for ∞. The amplification products were cleaned up using Ampure XP beads and normalized into a final pool with an Eppendorf liquid handling robot. Libraries were sequenced as part of a 150 bp paired-end sequencing run on the Illumina NextSeq platform following the manufacturer's protocol.

We used a custom pipeline that contained the following steps: joining pair-end reads; removing low-quality and chimeric reads; dereplication and dropping unique reads with low abundance; clustering OTUs; making OTU table; alignment; building a reference tree; and taxon assignment using FLASH (Magoc & Salzberg, 2011), the FASTX Toolkit (Hannon Lab, 2010) and the USEARCH pipeline (Edgar, 2010). See https://github.com/kstagaman/Process_16S and Goodfellow et al. (2019) for further details. We performed de novo OTU picking in UCLUST (Edgar, 2010), and sequences with 97% overlap were defined as belonging to the same bacterial OTU. To guard against sequencing errors, we filtered out OTUs with a frequency lower than 0.00005 as recommended (Bokulich et al., 2012).

Variation in Predictor and Outcome Variables

Of the females included in the analyses with behavioural predictor variables (Table A1), dietary dissimilarity (i.e. Sørensen diversity index) varied from 0 to 1, dissimilarity in relatedness calculated as their *R* value subtracted from 1 ranged from 0.31 to 1, and social connectedness (i.e. inverse path length or geodesic distance in the 1 m proximity network) varied from 0 to 1, where 0 represents unconnected dyads (Fig. A2).

In our full data set, mean unweighted UniFrac distances within the same season and year was 0.052 ± 0.004 for samples collected from the same individual ($N = 4$ samples) and 0.205 ± 0.042 for samples collected from different individuals within the same season and year ($N = 61$ samples). The low amount of within-individual variation in comparison to the between-individual variation suggests that one sample per individual is representative of its gut microbiome during that season and sufficient for analysis of beta diversity. Furthermore, the beta diversity of matched samples from the same adult female in the 2007 wet season and the 2009 dry season ($N = 22$ samples from 11 females) was lower (0.152 ± 0.031) than the female's mean beta diversity with samples from a different female and year (0.183 ± 0.021) for all but one female, and there was a significant difference in beta diversity between samples from the same versus different females in this sample (Wilcoxon signed-rank test: $N = 11$ females, $P < 0.001$).

Table A1
Number of adult females (AF) present, sampled and omitted from data analyses with behavioural predictor variables

Year	Group	AF group size	AF sampled	AF omitted	Reason for omitting samples
2007	BS	4	4	0	
	DA	5	5	1	Incomplete dietary information
	NP	4	4	0	
	RT	6	5	0	
	SP	4	4	0	
	WW	9	9	0	
2009	BO	8	8	0	
	BS	6	1	1	Lacked samples from majority of AF
	DA	7	2	2	Lacked samples from majority of AF
	NP	5	3	3	Lacked samples from majority of AF
	OD	6	6	0	
	RT	7	2	2	Lacked samples from majority of AF
	SP	3	0	–	
	WW	7	5	0	

Table A2
The coefficient estimates with their 95% confidence intervals, standard errors and *z* scores for the fixed effects included in the two most well-supported models predicting unweighted or weighted UniFrac distances in the data sets for within-group and between-group dyads and between-group dyads only

Outcome variable	Fixed effect	Coefficient estimate	Lower 95% CI	Upper 95% CI	SE	*z*
Within-group and between-group dyads						
Unweighted UniFrac	m1: Intercept	-1.568	-1.607	-1.529	0.020	-79.370
	m1: Year	0.315	0.274	0.356	0.021	15.010
	m1: Social connectedness	-0.086	-0.098	-0.074	0.006	-14.290
	m2: Intercept	-1.569	-1.607	-1.530	0.020	-79.580
	m2: Year	0.317	0.276	0.358	0.021	15.050
	m2: Social connectedness	-0.082	-0.095	-0.068	0.007	-12.040
	m2: Diet	0.009	-0.004	0.021	0.006	1.340
	m2: Relatedness	0.002	-0.008	0.013	0.005	0.410
Weighted UniFrac	m1: Intercept	-1.621	-1.655	-1.587	0.017	-93.610
	m1: Year	0.203	0.156	0.250	0.024	8.400
	m1: Social connectedness	-0.041	-0.057	-0.025	0.008	-5.130
	m2: Intercept	-1.620	-1.654	-1.586	0.017	-94.180
	m2: Year	0.200	0.152	0.247	0.024	8.270
	m2: Social connectedness	-0.048	-0.065	-0.030	0.009	-5.330
	m2: Diet	-0.016	-0.032	0.001	0.008	-1.890
	m2: Relatedness	0.001	-0.013	0.015	0.007	0.170
Between-group dyads						
Unweighted UniFrac	m1: Intercept	-1.559	-1.602	-1.515	0.022	0.022
	m1: Year	0.335	0.290	0.380	0.023	0.023
	m1: Social connectedness	-0.046	-0.086	-0.006	0.020	0.020
	m2: Intercept	-1.560	-1.604	-1.516	0.022	-69.490
	m2: Year	0.337	0.292	0.382	0.023	14.600
	m2: Social connectedness	-0.046	-0.086	-0.007	0.020	-2.290
	m2: Diet	0.004	-0.009	0.017	0.007	0.590
	m2: Relatedness	-0.001	-0.013	0.012	0.006	-0.100
Weighted UniFrac	m1: Intercept	-1.651	-1.698	-1.604	0.024	-68.680
	m1: Year	0.234	0.176	0.292	0.030	7.920
	m1: Social connectedness	-0.087	-0.142	-0.032	0.028	-3.080
	m2: Intercept	-1.646	-1.693	-1.598	0.024	-67.990
	m2: Year	0.229	0.171	0.287	0.030	7.740
	m2: Social connectedness	-0.085	-0.140	-0.030	0.028	-3.010
	m2: Diet	-0.012	-0.031	0.006	0.009	-1.280
	m2: Relatedness	-0.009	-0.027	0.009	0.009	-0.990

Table A3
Operational taxonomic units (OTUs) in these phyla, families and genera are expected to be structured by sociality based on previous studies

Taxon	Socially structured in 2007	Socially structured in 2009	Reference
Actinobacteria:	X	X	Tung et al. (2015)
Bifidobacteriaceae:	NA	X	Tung et al. (2015)
Bifidobacterium	NA	**X**	Tung et al. (2015)
Coriobacteriaceae	X	X	Tung et al. (2015)
Bacteroidetes:	–	–	–
Bacteroidaceae:	X	(✓)	–
Bacteroides	X	✓	Amato et al. (2017)
Porphyromonadaceae:	NA	X	Goodfellow et al. (2019)
Parabacteroides	NA	✓	Goodfellow et al. (2019)
Firmicutes:	✓	(✓)	–
Clostridiaceae	–	–	–
Clostridium	X	✓	Amato et al. (2017)
Eubacteriaceae	–	–	–
Eubacterium	X	(✓)	–
Lachnospiraceae:	✓	(✓)	–
Coprococcus	X	✓	Goodfellow et al. (2019)
Lachnospiracea incertae sedis	X	(✓)	–
Roseburia	✓	X	Amato et al. (2017)
Peptococcaceae:	X	(✓)	–
Desulfurispora	**X**	(✓)	–
Ruminococcaceae	X	(✓)	–
Flavonifractor	X	(✓)	–
Subdoligranulum	NA	(✓)	–
Streptococcaceae:	NA	–	–
Streptococcus	NA	NA	Amato et al. (2017)
Veillonellaceae:	X	X	Tung et al. (2015)
Propionispira	**X**	(✓)	–
Centipeda	**X**	(✓)	–
Fuscobacteria	NA	**X**	Tung et al. (2015)
Fusobacteriaceae	NA	**X**	Tung et al. (2015)
Fusobacterium	NA	**X**	Tung et al. (2015)
Proteobacteria:	–	–	–
Desulfovibrionaceae	–	–	–
Desulfovibrio	**X**	(✓)	–
Enterobacteriaceae	**X**	X	Tung et al. (2015)
Tenericutes:	X	X	Tung et al. (2015)
Anaeroplasmataceae	–	–	–
Asteroleplasma	**X**	(✓)	–
Mycoplasmataceae	NA	**X**	Tung et al. (2015)
Mycoplasma	NA	**X**	Tung et al. (2015)

Predictions were supported ✓; not supported X; no prediction made but structured in our data set (✓); or no prediction made and not structured in our data set (–). Bold symbols indicate rare taxa ($N < 3$ OTUs). NA denotes taxa not present in our data set.

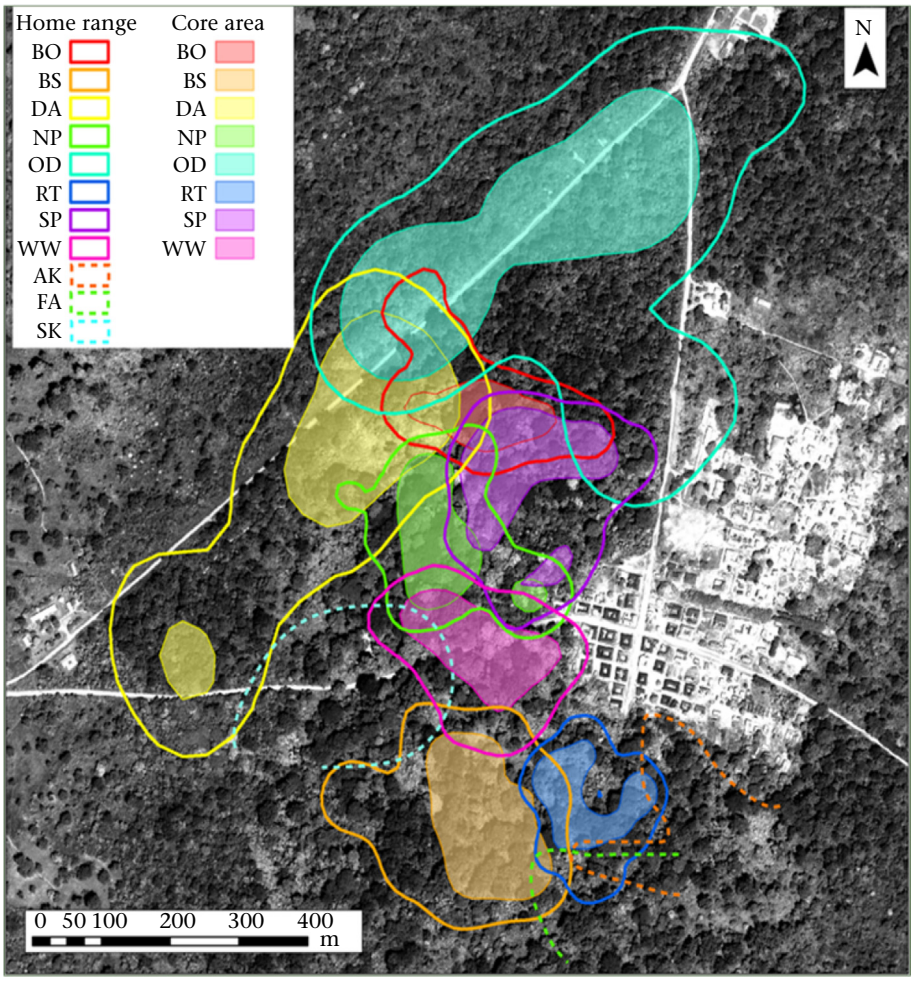

Figure A1. Home ranges and core areas for groups in the main forest fragment at Boabeng-Fiema, Ghana. Solid lines indicate home ranges of groups from which we collected behavioural data. Dashed lines indicate partial home ranges from other groups present in this forest.

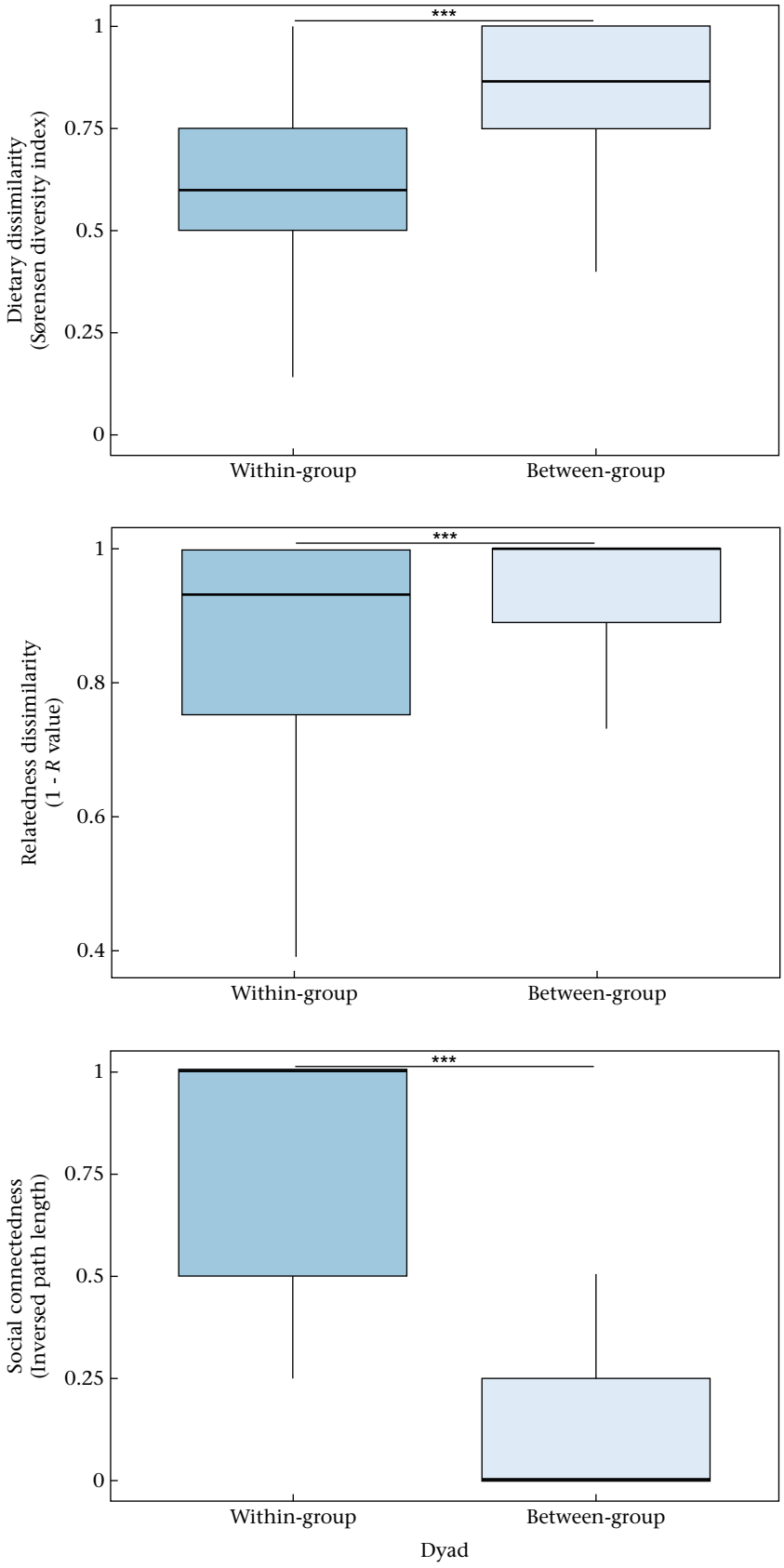

Figure A2. Comparison of dietary dissimilarity, relatedness dissimilarity and social connectedness for within-group and between-group female–female dyads (Wilcoxon signed-rank tests: $N = 88$ samples, all $P < 0.001$).