Social Behavior and Gut Microbiota in Wild Red-bellied Lemurs (*Eulemur rubriventer*) — In Search of the Role of Immunity in the Evolution of Sociality

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May 2015
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1. Introduction

1.1. Gut microbiota and the holobiome approach

Microbial communities living in the gut, skin and glands of vertebrates are of growing scientific interest, due to their emerging role as a functional link between the individual and its surrounding ecosystem. Recent developments in molecular methods have inspired a great amount of research on mutualistic microbiota in all taxa. In fields ranging from endocrinology and physiology to behavioral and evolutionary ecology, this new "holobiome approach" (Rosenberg et al. 2007) focuses on the reciprocal and dynamic relationships of mutualistic microbiota and their host.

Most of our current knowledge on gut microbiota comes from human medical research focusing on pathogens, while the mutualistic microbiota of wild vertebrate species has received much less attention. Furthermore, while the effects of microbiota on health are increasingly acknowledged, current research is for the most part lacking evolutionary perspective on this topic (but see Stilling et al. 2014). It is known, however, that vertebrate gut microbiota have an important influence on individual immunity (reviewed by Hooper et al. 2012) and is needed for providing enzymes for digestion (Mackie 2000). For example ruminants are known for their bacteria-rich rumen that slows down the passage of food material to enable efficient fermentation of cellulose by mutualistic microbes (Mackie 2000). New evidence also suggests that consistent with the "fermentation hypothesis" (Albone et al. 1974, Albone 1990), microbiota, especially that living in mammalian scent glands, affects social recognition by synthesizing pheromones (Sin et al. 2012, Theis et al. 2012, 2013, Douglas & Dobson 2013). In addition to this potential to alter behavior indirectly, an organism’s microbiota is known to have direct effects on its mood and behavior (Bravo et al. 2011, Ezenwa et al. 2012). The direct effects of gut microbes on behavior likely happen through Microbe-Gut-Brain (MGB)- axis, a physiological pathway involving the vagus-nerve (Bravo 2011, Montiel-Castro et al. 2013).

Mutualistic microbes play a notable role in an animal’s phenotype, but the determinants of individual’s microbiota are scantly known. Studies of wild species,
laboratory rodents and humans yield inconsistent results regarding the relative importance of genetic background, maternal transmission, and subsequent environmental contacts in determining gut microbial composition since all these factors can have a major impact on the microbiota (Penders et al. 2006, Lanyon et al. 2007, Khachatryan et al. 2008, Turnbaugh et al. 2009a,b, Benson et al. 2010, Degnan et al. 2012, Ubeda et al. 2012, Stevenson et al. 2014, Leclaire et al. 2014). Firstly, it is known that the composition of microbiota can have a genetic basis. For example, immunity-related genes are responsible for selectively enabling the establishment of certain microbes in the body, and thus form the physiological basis for microbiota in mice (Lanyon et al. 2007, Zomer et al. 2009, Benson et al. 2010). On the other hand, relatedness within human families does not predict similarity of gut microbiota (Turnbaugh et al. 2009b). Secondly, the microbes that colonize an animal body come primarily from the mother. Many gut microbes cannot live outside the host body, and must rely entirely on maternal transmission (reviewed by Funkhouser & Bordenstein 2013). Thus, as with many phenotypic traits, early life is important in determining microbiota. For example birth (Mandar et al. 1996) and early postnatal exposure by nursing (Jin et al. 2011, Fernandez et al. 2013) or even by eating soil or feces (Troyer 1984b, Soave & Brand 1991) have been considered the most important periods of transmission determining individual microbiota. In addition, recent evidence suggests that part of the microbiota can be acquired prior to birth (Funkhouser & Bordenstein 2013). Lastly, gut microbial composition is nevertheless known to fluctuate with changing diet, hormonal profiles, season and environment, hence highlighting the environmental impact on microbiota. While some of these effects are due to a changing diet and the abiotic environment, social environment is likely an important factor as well. Thus a mixture of vertical (genetic, maternal) and horizontal (social, environmental) transmission most likely determine individual microbiota. For example, both spotted hyenas (Crocuta crocuta) and chimpanzees (Pan troglodytes) have been found to have more similar microbiota within groups due to shared environment and social contact, but immigrant individuals still retained a slightly different microbiota after years of co-living (Degnan et al. 2012, Theis et al. 2012)
1.2. Social behavior and microbe transmission in a social group

Microbial transmission has been mainly studied in the context of pathogens and parasites. It is known that one important factor affecting pathogen or parasite transmission is social contact and this is considered one of the main costs of social life (Alexander et al. 1974, Möller et al. 1993, Altizer et al. 2003). For example vulnerability to infections increases with increasing group size in insects (Turnbull et al. 2011). However, it is less often suggested that in addition to entailing immunological challenge, sociality might actually also enhance immunity through transmission of beneficial microbes (Troyer et al. 1984a, Lombardo 2008, Archie & Theis 2011, Gilbert 2015). This is an important and understudied point of view when balancing the pros and cons of social lifestyle to understand the evolution of sociality. To sketch the role of gut microbiota in the evolution and social dynamics of social species, one needs to pay attention to three important aspects of the microbiota in a social context: diversity (affects immunity), similarity between individuals (affects recognition and immunity) and the presence of certain bacteria with known behavioral impacts.

Social behavior and diversity of microbiota

Many social behaviors include physical contact and can function as potential pathways for microbial transmission. For example, Kulkarni & Heeb (2007) experimentally infected zebra finches (Taeniopygia guttata) with Bacillus licheniformis bacteria and followed how the infection spread in their social group; preening and sexual behaviors transmitted the bacteria to all group members in less than a day. Similarly, frequent intimate kissing enhanced mutual transmission of mouth microbiota in humans (Kort et al. 2014). Behavior can affect microbial transmission and recent findings suggest that microbiota can affect host behavior as well (Ezenwa et al. 2012, Montiel-Castro et al. 2014). For example germ-free mice were found to be more active and less anxious than mice with normal microbiota (Heijtza et al. 2011). Similarly, autism-related lack of prosociality is associated with altered microbiota (Ming et al 2012, Cao et al. 2013).

Many authors have suggested that pro-social and affiliative behaviors, such as grooming, licking, preening, kissing or socio-sexual behaviors, might have partly evolved to serve beneficial microbial transmission (Troyer 1984, Lombardo et al. 2008, Ezenwa et al. 2012, Montiel-Castro et al. 2013). In social groups, exchanging mutualistic
microbes between multiple individuals allows more diverse microbiota to be present. Diverse microbiota in turn has been long suggested to be a requisite for a resilient immunity (Hooper et al. 2012, Lozupone et al. 2012), in the same way biodiversity makes ecosystems more resilient to change (Levine et al. 1999, Gunderson 2000). For example, socially transmitted microbiota was found to protect bumblebees (*Bombus terrestris*) from a common parasite (Koch & Schmid-Hempel 2011). While sociality brings immune challenges in the form of increased exposure (infection from others) and even susceptibility (immunosuppressive social stress) to similarly transmittable parasites (Alexander 1974, Möller et al. 1993, Altizer et al. 2003, Turnbull et al. 2011), transmission of beneficial bacteria might compensate this. This is an important issue for the theories of the evolution of sociality. Because mutualistic microbes can affect behavior and social behavior serves as a transmission route for these microbes, a positive feedback loop of increased sociality and microbial transmission is a possible mechanism in the evolution of sociality. This could help explain the association between sociality and health found in humans and other animals (House et al. 1988, Cohen et al. 2003, Sapolsky et al. 2004, Holt-Lunstad et al. 2010, Archie et al. 2014).

**Social behavior and similarity of microbiota**

Many group-living animals use friendly and affiliative behaviors to enhance social bonds or group cohesion and to avoid or reconcile conflicts between group-members (Vasey 1995, Aureli et al. 2002, Wittig et al. 2008, Hohmann et al. 2009, Radford 2011). When microbes are transmitted through social behavior in a social network, individuals most closely socially linked can be expected to share the same microbes. Mutualistic microbiota in social systems have received relatively little attention, whereas transmission patterns of parasites in social networks are well studied (Drewe et al. 2010, Godfrey et al. 2010, Griffin & Nunn 2011, Fenner et al. 2011, Bull et al. 2012, MacIntosh et al. 2012, Rimbach et al. 2015). For example, MacIntosh et al. (2012) found that female Japanese macaques (*Macaca fuscata yakui*) with more central position in a social network, and hence more grooming partners, had more endoparasites. Consistent with this, male social role predicted gut microbial composition in meerkats (*Suricata suricatta*, Leclaire et al. 2014). When social relationships are symmetric, transmission leads to more similar microbiota between social partners. For example human family members living together share more similar gut microbiota with each other than with
people outside the family, regardless of relatedness (Turnbaugh et al. 2009b, Song et al. 2013). Actually, even dogs share more similar gut microbiota with their human owners than other dogs outside the family (Song et al. 2013). Such group-specific microbiota has been found in yellow baboons (Tung et al. 2015), gorillas (Gomez et al. 2015), hyenas (Theis et al. 2012), meerkats (Leclaire et al. 2014) and chimpanzees (Degnan et al. 2012). Similar microbial compositions in social groups have been suggested to play a role in chemical communication, since mutualistic bacteria are responsible for synthesizing most of the odors and pheromones used in recognition by a variety of species. For example in spotted hyenas, group members share a “scent mark signature”, a similar scent mark chemical composition created by a similar scent gland microbial community (Theis et al. 2012).

The fact that individuals differ in their social personality (English et al. 2010, Koski 2011, Seyfarth et al. 2012, Neumann 2013, Carter et al. 2014) and possibly underlying physiology, speaks for a fluctuating balance between the immunological benefits and challenges of social life. This balance determines for example the relative values of microbial diversity and similarity under selection. Even though gaining a diverse microbial community through social contact might be beneficial, contact with individuals with too dissimilar microbiota might be harmful, because individuals become co-adapted to their microbes and unfamiliar bacteria might be pathogenic in a non-adapted individual (Feng et al. 2011, Stilling et al. 2014). In line with this, Barribeau et al. (2012) concluded that tadpoles most likely prefer the company of individuals with similar MHC-genotype as well as similar microbiota. Furthermore, such avoidance of opportunistic pathogens might be the reason behind mate choice driven by similar microbiota. For example, Drosophila melanogaster males were observed to mate more readily with females who had had the same diet and similar gut microbiota (Sharon et al. 2010).

**Social behavior and presence of certain beneficial microbes**

Gut microbiota consists of a variety of bacteria, of which some are more relevant for social behavior and transmission than others. Microbes that vary little in abundance and are shared more or less by all individuals of a given species are thought to form the “Core Microbiota” (Turnbaugh et al. 2007, but see also Lozupone et al. 2012). Moreover, core microbiota can be thought to consist of the maternally transmitted part of
microbiota. In addition, some microbes are more easily socially transmittable and highly variable in abundance (here termed as “Peripheral Microbiota”). For example in a recent study, Tung et al. (2015) found that a specific set of “socially structured bacteria” were transmitted through social interaction, leading more similar microbiota between social partners. One important genus among these socially structured bacteria was *Bifidobacterium*, which is associated with many health benefits in humans (Turroni et al. 2008). Another bacterial lineage with emerging importance in social behavior is *Lactobacillus*. Recent studies have revealed that *Lactobacillus* bacteria are associated with decreased anxiety in mice (Bravo et al. 2011), possibly due to their capability to promote oxytocin secretion (Poutahidis et al. 2013).

1.3. Stress and microbiota

Social behavior and microbiota seem to be more intimately linked than previously thought. One factor entwined with this relationship is stress physiology since stress affects both social behavior and microbiota. Stress is known to affect physiological homeostasis and to result in more or less social behavior depending on the context. Specifically, the effects of social stress can differ from the effects of non-social stress. The evolution of social life has created a whole new niche for the hormonal stress response, mediated by the hypothalamic-pituitary-adrenal axis (HPA). Whereas environmental stress can reduce social proximity (Barbosa & da Silva-Mota 2009, Overdorff & Tecot 2006, Tecot 2013), social stress can in fact enhance affiliation aimed at reducing stress and avoiding conflicts (DeVries et al. 1996, Carter 1998, Engh et al. 2006, Stöwe et al. 2008). It is clear that stress can affect microbial transmission through its effects on social behavior, but glucocorticoid stress hormones also have direct effects on microbiota. Hormonal profiles are associated with microbiota in general, which is evident in microbiota differences between the sexes (Alexy et al. 2003, Saag et al. 2011, Markle et al. 2013, Leclaire et al. 2014), but glucocorticoid stress hormones have especially fast and profound effects on gut microbes. In mice and primates, stress is known to reduce gut microbial diversity (Bailey & Coe 1999, Bailey et al. 2010), reduce the relative amount of lactobacilli (Bailey & Coe 1999, Galley et al. 2014, Galley & Bailey 2014) and change relative abundances of resident bacteria in general (Park et al. 2003). In humans, stressful time periods with higher cortisol levels are associated with changes in gut microbiota, most importantly reduced lactobacilli (Lizko et al. 1987,
Knowles et al. 2008). However, stress effects on microbiota are not unidirectional; through the vagus nerve of the gut-brain-axis (Bravo et al. 2011, Montiel-Castro et al. 2013) microbiota is known to affect HPA-axis function as well. For example, lack of functional microbiota sensitizes mice to stressors and increased HPA-axis reactivity and anxiety-like behavior (Sudo et al. 2004, Crumeyrolle-Arias et al. 2014).
Figure 1 depicts the relationships between microbiota, immunity, social behavior and stress physiology. This holobiomic map links an individual to its surrounding ecosystem, blurring the boundaries of individuals as functional units of nature. Some interactions, such as those between immunity and microbiota or stress physiology and social behavior, are extensively studied, but other interactions, such as those between social behavior or stress physiology and microbiota or stress physiology and microbiota, call for more research before we can draw an extensive map of these interactions.

1.4. Aims

To shed light on the interactions of gut microbiota, social behavior and HPA-axis, I collected data about social behavior, taxonomic composition of gut microbiota and fecal cortisol metabolites from a wild population of red-bellied lemurs (*Eulemur rubriventer*) in the Ranomafana National Park, eastern Madagascar. I ask whether patterns of social contact (group-membership, group size, social network) are associated with patterns of gut microbial composition (*Similarity*) and whether individual differences in sociality are related to specific aspects of gut microbiota (*Diversity, Presence of certain microbes*). Furthermore, I address the question of genetic vs. environmental determinants of microbiota by comparing gut microbial composition of family members with different relatedness. Lastly, I examine correlations between fecal cortisol levels and gut microbial composition to take the first step towards relating HPA-activity and gut microbial composition in this species and to raise further questions about the origin of possible covariance.

Red-bellied lemurs are cooperative and highly social primates. Individuals vary in how much they contribute to social actions and with whom. For example individual significantly differ in how much allomaternal care they provide to infants (Tecot & Baden, unpublished data). By using social network analysis and by constructing individual sociality indices from the amount of social behaviors an individual exhibits, I examine the intra-group differences in social behavior. My overall hypothesis is that the amount and direction of close social interaction is positively correlated with gut microbial diversity and similarity, respectively. This pattern should be visible when comparing i) individuals with different levels of social interactions in the same group, ii)
individuals between groups (no interaction), and iii) individuals from groups of different sizes. In addition, the association between gut microbiota and social behavior could partly be mediated by interaction with the stress hormones of the HPA- axis. To test this hypothesis, several specific predictions can be made:

1. Gut microbial composition should be more similar between group-members than between individuals from different groups.
2. Gut microbial diversity should be greater in larger groups.
3. More social individuals should have more diverse gut microbiota than less social individuals.
4. Pairs with short distance in a social network (frequent mutual interaction) should have more similar gut microbial composition than pairs with a greater distance (rarely interact with each other).
5. Gut microbial composition and fecal cortisol metabolites should covary. Specifically, high fecal cortisol levels should be associated with low gut microbial diversity.

In addition to social life, I investigate whether genetic relatedness and early environment within group are determinants of gut microbiota in this species. To test this hypothesis I predict the following: If genes are more important than the social environment in determining gut microbiota, adults should have more similar gut microbial composition with their offspring than with each other.

1.5. **Study species and study site**

I carried out the behavioral observations and fecal sample collection in the Ranomafana National Park, Eastern Madagascar, from August 2013 to February 2014. The area is a rainforest with topographic variation, montane cloud forest growing on ridges and lowland rainforest in valleys (Wright 1999). My specific study site, Vatoharanana, is a high altitude region located 5 km south of the Centre ValBio research station, by the edge of the forest. There are at least 12 lemur species living in the area.

Within this study area, the focal study subjects were eight family groups of red-bellied lemurs (*Eulemur rubriventer*). Additional fecal samples were collected from other groups when encountered (total 36 individuals). Red-bellied lemurs are frugivorous strepsirrhines, living in small, relatively stable, monogamous family units of 2-6
individuals with one breeding pair and their offspring of different ages (Overdorff 1996b, Overdorff and Tecot 2006, Tecot 2008). They are an egalitarian species that exhibit allomaternal care and have a seasonal breeding cycle (Tecot 2008, 2010). Some fathers and juveniles help to raise infants and all family members participate in social interactions, such as grooming, huddling or playing with each other. Groups of red-bellied lemurs have fixed territories that vary in their fruit availability during the year (Overdorff 1996a, Tecot 2008). My data were collected during the time of the year when infants were born and fruit availability was generally low (Tecot 2008), though within this season the diet of the animals changed many times depending on which particular trees were fruiting. Furthermore, because of the general fruiting patterns of the food plants, all study individuals had a relatively similar diet at any given time.

2. Materials and Methods

2.1. Collection of Behavioral Data

There were four people collecting behavioral data in our field team. Two of us were local field technicians with extensive expertise in lemur behavioral and hormonal research. We followed the eight family groups of red-bellied lemurs in the Vatoharanana area opportunistically: Whenever a group was found, it was followed for the rest of the day. I kept track of group size and composition and of any changes therein. Continuous behavioral data were collected from 27 focal individuals in eight groups, by following each group one whole day opportunistically every time they were found (approximately seven hours per day, every on or two weeks). Behavioral data were obtained by using scan sampling method with five-minute intervals; every five minutes, each group member was classified to one behavioral category of the Red-Bellied-Lemur Allomaternal Care Project ethogram (Table 1; Tecot & Baden, unpublished field manual). For social behaviors (Mutual Grooming, Huddling, Playing), I recorded with whom the behavior was exhibited. Because four different people participated in the behavioral classification, inter-observer-reliability was tested repeatedly (Gwet 2001). We accumulated 15-150 hours of behavioral data from each
Only the groups with more than two individuals and more than 50 hours of behavioral data were used to investigate questions related to social behavior.

Table 1. Ethogram, Tecot & Baden, unpublished field manual

<table>
<thead>
<tr>
<th>Mark</th>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Move</td>
<td>Moving inside a single tree (less than 3 meters)</td>
</tr>
<tr>
<td>T</td>
<td>Travel</td>
<td>Directed movement more than 3 meters</td>
</tr>
<tr>
<td>R</td>
<td>Rest</td>
<td>Animal is motionless (eyes open or closed)</td>
</tr>
<tr>
<td>HUD</td>
<td>Huddle</td>
<td>Resting in physical contact with others, tails wrapped around each other</td>
</tr>
<tr>
<td>PPLY</td>
<td>Partner Play</td>
<td>Disoriented non-continuous playful behavior between more than one individuals</td>
</tr>
<tr>
<td>SPLY</td>
<td>Self-play</td>
<td>Disoriented non-continuous playful behavior not directed to other individuals</td>
</tr>
<tr>
<td>SGR</td>
<td>Self-groom</td>
<td>Grooming one’s own fur</td>
</tr>
<tr>
<td>AGR</td>
<td>Allogroom</td>
<td>Grooming another individual</td>
</tr>
<tr>
<td>RGR</td>
<td>Receiving Groom</td>
<td>Receiving groom from another individual</td>
</tr>
<tr>
<td>MGR</td>
<td>Mutual Groom</td>
<td>Two individuals grooming each other simultaneously</td>
</tr>
<tr>
<td>FD</td>
<td>Feed</td>
<td>Handling, sniffing and eating food items</td>
</tr>
<tr>
<td>AGG</td>
<td>Aggression</td>
<td>Fast and loud chasing or cuffing other individual</td>
</tr>
<tr>
<td>AGM</td>
<td>Scent marking</td>
<td>Ano-genital scent marking behavior</td>
</tr>
</tbody>
</table>

Table 2. Amount of behavioral data per group and the criteria used to choose groups to study social behavior. All groups were used in other analyses described in the methods

<table>
<thead>
<tr>
<th>Group</th>
<th>Group Size</th>
<th>Hours of behavioral data</th>
<th>Used to study behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>60</td>
<td>X</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3-4</td>
<td>140</td>
<td>X</td>
</tr>
<tr>
<td>8</td>
<td>4-6</td>
<td>150</td>
<td>X</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>130</td>
<td>X</td>
</tr>
<tr>
<td>53</td>
<td>3</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>
Constructing sociality indices and interaction matrices

Individual sociality indices were constructed based on the behavioral scan sample data. They were calculated as the proportions of time each subject spent in particular social behaviors. Specifically, I constructed two indices for two different social behaviors, respectively:

1. **Huddle index**: proportion of time spent huddling with others out of the total amount of rest time.

2. **Social groom index**: proportion of time spent socially grooming (mutual groom, allogroom, receiving groom) out of the total amount of time observed.

To construct interaction matrices, I used a standard method for social network analyses (Whitehead 2008). Pairwise comparisons of mutual interactions yielded social distance values for each pair of individuals. Distance values were calculated for a pair of individuals as the proportion of time spent in mutual interaction out of total time spent in social interaction of the two individuals in each pair. Interaction matrices were constructed for two social behaviors:

1. **Huddle matrix**: a distance matrix of how much each pair spent huddling together

2. **Groom matrix**: a distance matrix of how much each pair spent grooming each other

Sociality indices and interaction matrices were constructed separately for two time periods: 1) before infants were born (September-October in 2013), and 2) and after infants were born (December 2013 until January 2014).

2.2 Fecal Sample Collection and Analysis for Cortisol Metabolites

I collected fecal samples for hormonal assays by wrapping them in foil and drying them by fire (Tecot 2001, 2008, 2013). Samples were kept dry by storing them in a bag full of silica pearls. Hormonal samples were collected from individuals of the focal groups when ever possible to detect changes in hormonal status. Samples were kept dry and brought to the Laboratory for the Evolutionary Endocrinology of Primates (LEEP) in University of Arizona. Fecal samples were ground, sifted and 0.1 g was extracted from each sample into a solution of half ethanol half water. Samples were then mixed
(vortexed), centrifuged and the supernatant was poured off. One ml of each sample was taken into new tubes and 4 ml of ethyl acetate was added to free conjugant steroids. Samples were mixed and centrifuged again and the top layer was aspirated into culture tubes, evaporated in water bath after which 1 ml of 30 % methanol was added. Samples were conditioned with 1 ml of methanol and 1 ml of water (distilled). 1 ml of sample was added and washed with 5 % methanol. Steroids were eluted from the column with 2 ml of methanol, which was then evaporated and replaced with 1 ml of ethanol.

Cortisol assays were done using an enzyme immunoassay (EIA) protocol by Coralie Munro: Enzyme-labeled antigen (horseradish peroxidase cortisol) was diluted in EIA-buffer (1: 100 000) and mixed. 0.5 ml of samples were first evaporated and 0.3 ml of the antigen dilution (T:HRP-EIA) was added to all samples and standards. Samples and standards were then mixed and transferred to assay tubes. For control, 0.3 ml pure EIA-buffer was added to NSB-tubes and 0.3 ml T:HRP-EIA was added to B0-assay tubes. Plates coated with steroid conjugate were washed and 0.1 ml of each sample and standard mixture were plated in duplicate, shaken and incubated for 2 hours in a humidity chamber to enable binding. Plates were then washed to separate bound and free cortisol. 1 ml substrate was added, the plate was mixed for five minutes and absorbance was calculated with a Biotek plate reader. The slope of serially diluted fecal extracts was parallel to that of the cortisol standard, \( F = 0.12(11,12) \), \( p = 0.730 \). Fecal cortisol assays were determined to be accurate (96.68 ± 1.79% standard error of the mean).

2.3 Fecal sample collection and gut microbial DNA analysis

I collected fecal samples for microbial DNA analysis in Eppendorf tubes filled with RNAlater. Minimum of one sample was collected from all focal individuals. Second samples for seasonal comparison were collected from 15 individuals in five groups. To increase sample size, I collected additional samples opportunistically whenever encountering individuals from non-focal groups. Even though I did not have matching behavioral data for these samples, they were used in the analysis of demographic correlates, such as with age, sex or group size. Altogether 110 microbial fecal samples were collected.
Eppendorf tubes were kept in a freezer (-80°C) for 6 months before sending them to the Knight Laboratory in the University of Colorado to be analyzed. DNA-extraction was done using MOBio PowerSoil –kits. PCR was run with primers targeted at V1-V3 region of the 16S rRNA gene. The resulting amplicons were sequenced using Illumina MiSeq platform. I used QIIME software to match barcodes with samples, to eliminate bad quality DNA and to flip reverse sequences (Caporaso et al. 2010). Here, quality filtering was done with default settings of the "split_libraries_fastq.py" command (Q>20, 1% risk marginal in bases). Sequences were clustered and matched with known taxonomic database (Greengeens) using open reference OTU picking (pick_open-reference_otus.py) while enabling reverse strand matching. This way the list of the known OTUs (Operational taxonomic unit, comparable to "species") that matched with the database could be enriched with the de novo clustered unknown OTUs. This is important, because a significant proportion of gut microbiota, especially that of lemurs, belongs to these unknown taxa (Katherine Amato, personal communication). Here for example, open reference OTU picking yielded 1,131,454 reads (sequences) whereas closed reference OTU picking (without de novo clustering) yielded only 304,163 reads. The amount of samples that were successfully sequenced and had a minimum of 3,000 reads was 92. The data was rarefied to 3,000 reads per sample.

### 2.2. Statistical analyses

**Describing microbial data**

I performed statistical analyses using R (R version 3.1.2, "Pumpkin Helmet", R Core Team 2014). The microbial data were first standardized as relative abundances (the frequency of counts of each OTU relative to the total count) and converted into dissimilarity indices. The used dissimilarity indices describe the relative differences of microbial species communities between individuals based on presence–absence information (Sørensen dissimilarity index) or relative abundance (Bray–Curtis dissimilarity index) (Legendre & Legendre 2012). The Bray–Curtis dissimilarity index is an index used to calculate the dissimilarity of species composition between two sites (here, between samples). It is calculated as follows:

\[
\text{BC}_{\alpha\beta} = 1 - \frac{2c_{\alpha\beta}}{s_{\alpha} + s_{\beta}},
\]

where \(c_{\alpha\beta}\) is the sum of the minimum of counts of OTUs between samples, and \(s_{\alpha}\) and \(s_{\beta}\) are the total counts of each sample.
where $C_{\alpha\beta}$ is the sum of the lesser counts of each OTU present in both samples $\alpha$ and $\beta$, and $S_\alpha$ and $S_\beta$ are the total number of OTUs present in both samples. The Sørensen dissimilarity index is a similar index, but unlike Bray–Curtis, it only applies for presence–absence data. It is calculated as follows:

$$Q_S = \frac{2C}{A+B},$$

where $C$ is the absolute number of OTUs shared by the two samples, and $A$ and $B$ are the total number of OTUs in each samples, respectively. I used these two different indices to get an overview of gut microbial dissimilarity based on both (a) having different OTUs present or absent (Sørensen), and (b) having different relative abundances of OTUs (Bray–Curtis). Sørensen indices were also used to reinforce Bray–Curtis metrics of dissimilarity, because differences in the presence–absence of certain bacteria are a more reliable measure of dissimilarity of microbial communities than differences in relative abundances. In addition to dissimilarity indices, I used Shannon diversity index and richness to describe the gut microbial diversity.

**Clustering of microbial data**

I used dissimilarity indices to construct dissimilarity matrices of all individuals, and these were used to cluster data to reveal the distribution of gut microbiota across the study population. Clustering from the dissimilarity matrices was done using the $k$-means method, which is an algorithm that assigns data points to clusters by minimizing the sum of squared distances of points to the center of each cluster (Legendre & Legendre 2012). Eight clusters were found using this method, which coincides with the number of study groups.

Since the monthly changes in the gut microbiota were small (controlled for individual and pregnancy, $R^2 = 0.02$, $p = 0.012$), I pooled the samples from two months before and after infants were born (September–October and December–January) to form two comparable time periods (Seasons). Clustering was then done on a subset of the data containing only one sample per individual, all taken during the first season (Sep–Oct). Here, clustering was done with a defined number of eight clusters, derived from the observed clustering pattern of the whole data. This was further visualized using principal coordinate analysis (PCoA), which provides a good overview of the data.
distribution before any further analyses. PCoA aims at finding the axis along the multidimensional distribution that exhibits the most variation (Legendre & Legendre 2012); this axis is called the first principal coordinate.

**Analyses using dissimilarity indices**

To test the effect of time (month and season) and pregnancy on the microbiota composition, I used permutational multivariate analysis of variance using dissimilarity indices as distance matrices. This analysis was performed with the `adonis` function as implemented in the vegan package in R (Oksanen et al. 2014). Permutational multivariate analysis was chosen because the elements in a dissimilarity matrix are not independent. This test is based on comparing the observed association between dissimilarity indices and other variables with those arising when the index values are repeatedly randomly permuted. This allows reliable estimation of statistical significance. The same test was used for the smaller subset of data with one sample per individual collected in the first season to test for the effects of age, sex, group and group size. Similarly, I compared samples of a set of females and their mates before and after pregnancy to test for the possible effects of pregnancy, and used principal coordinate analysis to visualize this.

**Analyses using diversity and richness**

In addition to these, I used generalized linear models to test for the effect of season and sociality indices on gut microbial diversity and richness. This was done by fitting a line by linear regression the data (minimizing the sum of squared residuals) and calculating whether this regression explains significantly more of the observed variation that would be expected by chance, assuming a distribution of exponential family (Grafen & Hails 2002). Furthermore, to test correlations between interaction matrices (converted from social proximity to social distance matrices) and microbial dissimilarity matrices, I used the Mantel test of matrix correlation (Legendre & Legendre 2012). The Mantel test is a permutational test of correlation, where statistical significance is tested by repeatedly randomly permuting the rows and columns of one of the matrices, and the correlation coefficients are compared with that observed for the original matrices. $P$-value is calculated as a proportion of permutations that lead to higher than the observed correlation coefficient. For tests involving sociality indices and interaction
matrices I used data only from groups, for which there was sufficient behavioral data to construct these indices and matrices for both seasons (before and after infants were born). This subset of data consisted of 26 samples encompassing 13 individuals from four groups.

Which bacterial taxa are behind the trend?

To find out which bacterial taxa are responsible for a given trend, I used Dufrene–Legendre indicator analysis to calculate indicator values for each OTU in the groups of samples under comparison (Dufrene & Legendre 1997). The Dufrene–Legendre indicator value is calculated as follows:

\[ \text{IndVal}_{ij} = A_{ij} \times B_{ij} \times 100 \]

, where \( A_{ij} \) is the number of OTU \( i \) in class \( j \) (such as the family group) divided by the total number of OTU \( i \), and \( B_{ij} \) is the number of samples (individuals) in class \( j \) that contain OTU \( i \) divided by total amount of samples in class \( j \). The statistical significance of the resulting indicator values are then tested by random permutation. Here, \( p \)-value of 0.01 (except 0.05 when analyzing the effect of pregnancy with a small dataset) was used as a threshold for significance, and of each class, ten OTUs with the highest indicator values were chosen.

3. Results

3.1. Environmental, demographic and genetic factors affecting microbiota

The gut microbiota of red-bellied lemurs was dominated by the phyla Bacteroidetes, Proteobacteria and Firmicutes (Fig. 5). Within one season, gut microbiota clustered strongly according to the family group and the groups formed three bigger clusters partially paralleling the location of their territories (Fig. 2 and 3). That is, the groups in the two smaller clusters have neighboring or overlapping territories, but not all groups in the biggest cluster seem to be so closely located. Individual differences in gut microbial composition (by both dissimilarity indices) were best explained by group identity (see Table 3). In addition, within-group differences were significantly explained
by pregnancy (see Table 3). Additional analyses revealed that microbiota during and after pregnancy changed significantly in adult females ($R^2 = 0.16$, $p = 0.02$ with both dissimilarity indices; see Fig. 4), but not in a control group of the corresponding males ($p = 0.5$). This difference was not in the gut microbial diversity, but rather in differences in the species (OTUs) of the bacterial community.

Indicator analysis revealed that differences between the microbiota of the same females during and after pregnancy were driven by phylum-level changes. Specifically, pregnant females’ gut microbiota was characterized by bacteria belonging to phyla Proteobacteria and Verrucomicrobia, whereas post-pregnant females’ gut microbiota was characterized by abundance of bacteria belonging to phylum Firmicutes. Moreover, the best indicator species for group-specific differences in gut microbial composition were bacteria belonging to the order Clostridiales (in Phylum Firmicutes). Figure 5 summarizes group differences in gut microbial composition at the phylum-level. Group size, age, sex and age-sex interaction controlling for pregnancy had no significant effect on gut microbial composition (See Table 3).

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
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<td>0.23</td>
<td>2.39</td>
<td>0.19</td>
<td>&lt;0.01</td>
</tr>
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<td>0.12</td>
<td>0.12</td>
<td>1.27</td>
<td>0.03</td>
<td>0.13</td>
</tr>
<tr>
<td>Sex</td>
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<td>0.11</td>
<td>1.10</td>
<td>0.03</td>
<td>0.23</td>
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<tr>
<td>Group</td>
<td>7</td>
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<td>0.18</td>
<td>1.92</td>
<td>0.36</td>
<td>&lt;0.01</td>
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<td>Group Size</td>
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<td>0.08</td>
<td>0.87</td>
<td>0.02</td>
<td>0.65</td>
</tr>
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<td>Age:Sex</td>
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<td>0.08</td>
<td>0.82</td>
<td>0.02</td>
<td>0.76</td>
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<tr>
<td>Residuals</td>
<td>13</td>
<td>1.25</td>
<td>0.10</td>
<td></td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>3.62</td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
</tbody>
</table>
Figure 2. PCA-ordination of the filtered data. Gut microbes cluster as family groups (numbered colored areas) and these groups form three bigger clusters, partially paralleling their territory location (See Fig. 3).

Figure 3. Geographical territories of the study groups. Group colors are the same as in Fig. 2.
**Figure 4.** Breeding females’ gut microbial compositions in relation to each other (lines) and during and after pregnancy (colors) visualized with principal component analysis.

**Figure 5** Gut microbial composition of the eight study groups, allocated by phyla. White area covers unknown species.
3.2. Seasonal Change in Gut Microbiota

Seasonal change in the gut microbial composition was investigated with a dataset where breeding females had been removed to eliminate the strong effect of pregnancy. The relative abundances of gut microbes (Bray-Curtis dissimilarity index) varied between the two seasons significantly, though the effect was quite small (Table 4). Analysis using presence-absence data for microbial composition (Sørensen dissimilarity index) showed no significant effect of the season (Table 5). Gut microbial diversity (Shannon) and richness decreased significantly from before to after infants were born (p = 0.02 for richness, p = 0.04 for diversity, Fig. 5). A similar trend in diversity was not detected in breeding females when this was analyzed separately. Indicator analysis revealed that the trend of decreasing gut microbial diversity was associated with increasing abundance of bacteria belonging to the phylum Bacteroidetes, most importantly genus *Prevotella*.

**Table 4.** The effect of season on gut microbial composition using relative abundances (Bray-Curtis dissimilarity index). Because data were unevenly distributed in time (Different groups had different amount of data in different months), Month, Group and their interaction were added to the analysis as control variables before Season.

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>SS</th>
<th>MS</th>
<th>F-value</th>
<th>$R^2$</th>
<th>p</th>
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<td>1.50</td>
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<tr>
<td>Month</td>
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<td>0.32</td>
<td>4.24</td>
<td>0.17</td>
<td>&lt;0.01</td>
</tr>
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<td>Season</td>
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<td>0.16</td>
<td>2.07</td>
<td>0.08</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Group:Month</td>
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<td>0.12</td>
<td>1.61</td>
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<td>0.01</td>
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<tr>
<td>Residuals</td>
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<td>0.08</td>
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<td></td>
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<td>17</td>
<td>1.86</td>
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</table>

**Table 5.** The effect of season on gut microbial composition using relative abundances (Sørensen dissimilarity index). Because data was unevenly distributed in time (Different groups had different amount of data in different months), Month, Group and their interaction were added to the analysis as control variables before Season.

<table>
<thead>
<tr>
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<th>Df</th>
<th>SS</th>
<th>MS</th>
<th>F-value</th>
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<td>0.14</td>
<td>1.16</td>
<td>0.17</td>
<td>0.10</td>
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<tr>
<td>Month</td>
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<td>0.26</td>
<td>0.26</td>
<td>2.13</td>
<td>0.11</td>
<td>&lt;0.01</td>
</tr>
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<td>Season</td>
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<td>0.15</td>
<td>1.25</td>
<td>0.06</td>
<td>0.11</td>
</tr>
<tr>
<td>Group:Month</td>
<td>3</td>
<td>0.49</td>
<td>0.16</td>
<td>1.36</td>
<td>0.20</td>
<td>0.01</td>
</tr>
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<td>Residuals</td>
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<td>1.09</td>
<td>0.12</td>
<td></td>
<td>0.45</td>
<td></td>
</tr>
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<td>Total</td>
<td>17</td>
<td>2.41</td>
<td></td>
<td></td>
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</table>
3.3. Gut Microbial Composition and Interaction Matrices

There was no significant correlation between gut microbial dissimilarity (by Bray-Curtis or Sørensen dissimilarity indices) and social connectedness within group with any of the interaction matrices (Huddle matrix and Groom matrix before and after infants were born).

3.4. Gut Microbial Composition and Sociality Indices

Individuals varied in the amount of resting time they spent huddling together and the amount of time they spent grooming others (Fig. 7). Groom indices were significantly negatively correlated with gut microbial diversity between individuals through time ($p=0.01$). After adding time to the analysis, negative correlations were apparent in both indices (Table 6), but only before infants were born (Fig. 7, a, b). Richness followed a similar trend (Fig. 7, c, d), though it was not significantly correlated with the huddle index at any time. Overall, huddle index decreased ($p<0.01$) and groom index did not change from before to after infants were born.

Figure 6 Gut microbial diversity (left) and richness (right) are higher before than after infants are born
Table 6. Results of generalized linear model testing for the effects of both sociality indices on gut microbial diversity, after the effect of time (Pre/Post infant) is taken into account.

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>Std. Error</th>
<th>t value</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>(Intercept)</td>
<td>4.30251</td>
<td>0.09567</td>
<td>44.973</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pre/Post Infant</td>
<td>0.18386</td>
<td>0.07929</td>
<td>2.319</td>
<td>0.0317</td>
</tr>
<tr>
<td>Groom Index</td>
<td>-8.65217</td>
<td>3.37531</td>
<td>-2.563</td>
<td>0.019</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>Estimate</th>
<th>Std. Error</th>
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<th>p</th>
</tr>
</thead>
<tbody>
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<td>(Intercept)</td>
<td>4.4605</td>
<td>0.1764</td>
<td>25.287</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pre/Post Infant</td>
<td>0.3958</td>
<td>0.1143</td>
<td>3.462</td>
<td>0.00261</td>
</tr>
<tr>
<td>Huddle Index</td>
<td>-0.8527</td>
<td>0.3967</td>
<td>-2.149</td>
<td>0.0447</td>
</tr>
</tbody>
</table>

Figure 7 Gut microbial diversity and richness in relation to sociality indices. Before infants are born (in red), diversity is negatively correlated with groom index (a) and huddle index (b), but correlation is not apparent after infants are born (in black). Richness follows similar trends (c,d), but correlation with huddle index (d) is not significant.
3.5. Gut microbial composition and stress hormones

To control for the effects of pregnancy on HPA-axis activity, breeding females were excluded from the analysis of correlations between cortisol profiles and gut microbial composition. Fecal cortisol levels were positively correlated with gut microbial composition ($R^2 = 0.14$, $p = 0.05$ with Sørensen dissimilarity index, $R^2 = 0.14$, $p = 0.04$ with Bray-Curtis dissimilarity index). Dissimilarity indices correlated with each other ($p<0.01$), indicating that they speak for the same thing. After adding group and sex-age class into the analysis as control variables, the result was even more significant (Table 7). The effect of cortisol on gut microbiota was not related to diversity or richness, but rather to differences in the species of the bacterial community, specifically species belonging to phylum Firmicutes.

Table 7. Correlation of Cortisol levels (CORT) with gut microbial composition using relative abundances (Bray-Curtis dissimilarity index). Group and sex-age class are control variables.

<table>
<thead>
<tr>
<th>Df</th>
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<th>MS</th>
<th>F-value</th>
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<th>p</th>
</tr>
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<tr>
<td>Group</td>
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<td>0.56</td>
<td>0.11</td>
<td>1.74</td>
<td>0.49</td>
</tr>
<tr>
<td>Sex-Age Class</td>
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<td>0.23</td>
<td>0.12</td>
<td>1.80</td>
<td>0.20</td>
</tr>
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<td>CORT</td>
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<td>0.15</td>
<td>0.15</td>
<td>2.29</td>
<td>0.13</td>
</tr>
<tr>
<td>Residuals</td>
<td>3</td>
<td>0.19</td>
<td>0.06</td>
<td>1.14</td>
<td>0.17</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>1.14</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
</tbody>
</table>

4. Discussion

4.1. Environmental and Demographic Variation in Gut Microbiota

Group identity, age, and seasonal change were the main factors explaining variation in the gut microbiota of red-bellied lemurs, which was very similar between all individuals within one group. No effect of relatedness (differences in microbiota between parent-offspring, parent-parent and offspring-offspring pairs) was detected. The main factor explaining within-group differences in gut microbiota was pregnancy. In particular females gut microbiota differed from the other group member’s microbiota and breeding females also experienced a change in the gut microbial composition at the time of giving birth. Similarly to
these results, the microbiota of pregnant women (Collado et al. 2008, Koren et al. 2012, Romero et al. 2014) as well as well breeding female European badgers (Meles meles) were found to differ from non-breeding females’ microbiota. Moreover, paralleling the trend found by Koren et al. (2012) in pregnant women, our results show that pregnant females gut is abundant in bacteria belonging to the phylum Proteobacteria, while post-pregnant females’ guts are abundant in bacteria belonging to the phylum Firmicutes. Since birth and early environment are important in the development of a healthy microbiota, pregnancy-related modification of gut microbiota might be an adaptive way of readying the microbial environment for the infant (Koren et al. 2012). In addition to pregnancy, no other sex or age differences were found. This is quite surprising, given the number of studies indicating that microbiota can differ between sexes (Alexy et al. 2003, Saag et al. 2011, Markle et al. 2013, Leclaire et al. 2014) and tends to change during development until individuals reach maturity (Palmer et al. 2007, Mariat et al. 2009, Sin et al. 2012, Leclaire et al. 2014).

The dissimilarity of gut microbiota between family groups might be partially due to differences in the food sources and microhabitats of different territories. Geographical range has been previously linked with differences in gut microbiota in primates (Degnan et al. 2012, Moeller et al. 2013, Gomez et al. 2015) However, since the territories of our study groups have overlap, environmental differences seem an inadequate explanation for the observed dissimilarity of microbiota. Furthermore, red-bellied lemurs are frugivores and all individuals within the same area experience more or less synchronized changes in diet because of the fruiting patterns of their food plants. Despite this, the seasonal change in gut microbiota was not nearly as robust as differences between groups, suggesting that spatial variation in diet or environmental conditions cannot alone explain the differences between groups.

However, group-specific microbiotas formed three bigger clusters that might be explained by the location of group territories (Fig 3). Indeed, the groups in the two smaller clusters (8+ 11 and 5+10) have neighboring or overlapping territories, but not all groups in the biggest cluster (3+7+9+53) seem to be so closely located. This territory information is not based on GPS-coordinates, but a trail-locating system and might be slightly misleading. More specific information
of territories from various distances of each other is needed before the effect of territory distance on gut microbial composition can be described.

**4.2. Group-specific microbiota**

Group-specific gut microbiota reflects the social life of this species, with high within-group cohesion and extremely low interaction between members of different groups. These results add to the increasing evidence of group-specific microbiota in highly social species (Turnbaugh et al. 2009b, Song et al. 2013, Degnan et al. 2012, Theis et al. 2012, Leclaire et al. 2014, Tung et al. 2015, Gomez et al. 2015). Red-bellied lemurs, just like cooperatively breeding meerkats and hyenas, provide allomaternal care and rely on in-group cooperation in caring for the young. In this context, shared microbiota might be crucial for group-specific scent marks, recognition and bonding. More importantly, however, shared microbiota in a small group can be seen as a mechanism of immunity synchronization; individuals become accustomed to their gut microbes and indeed the same gut bacteria might be mutualistic in an accustomed individual but pathogenic as a novel infection (Barribeau et al. 2011, Feng et al. 2011, Stilling et al. 2014). Sharing microbiota makes it certain that everybody is accustomed to the same bacterial allies, and will not infect their group members with potential pathogens. In support of this hypothesis, cooperative breeders and at least one species with shared infant care are generally known to possess synchronized physiological processes within the group. For example, in many cooperatively breeding primates, other group members can reflect the hormonal status of the pregnant female (Storey et al., 2000, Nunes et al., 2001, Ziegler et al., 2004, Tecot 2008) possibly readying them to their task in providing allomaternal care (Ziegler 2000). Furthermore, cooperatively breeding marmosets (*Callithrix jacchus*) were found to have more similar personalities, and thus most likely more similar hormonal profiles (Koolhaas et al., 1999, Carere et al. 2010, Koolhaas et al., 2010 Sih 2011) than chimpanzees relying on dyadic rather than group-wide cooperation (Koski & Burkart 2015). To explore this further, synchronized hormonal patterns in cooperative groups needs to be studied more.
4.3. Microbial transmission in a social network

I found no correlation between social contact and similarity of microbiota within groups, but this is likely because family groups of this species are small (2-6 individuals) and social connectedness within group is relatively high between all individuals. However, group-specific gut microbiota speak for the importance of social contact in determining the microbiota in this species. Furthermore, even the breeding pair shared similar gut microbiota, which is most likely formed only through social or sexual contact, since they are not related and do not share early environment (i.e. they derive from different natal groups). This is interesting, because if social contact can function as a transmission route for gut microbes (Kulkarni & Heeb 2007, Kort et al. 2014, Tung et al. 2015), these microbes can carry information of social contact as well. In wild animal research, there is a strong bias in the study of social behavior towards diurnal and highly social species. Gut microbiota could be used to draw maps of social networks also in asocial, nocturnal or otherwise cryptic species, whose (social or sexual) behavior is hard to study in wild. Paralleling this idea, Zohdy et al. (2012) managed to capture the pattern of social contacts in wild nocturnal mouse lemurs (*Microcebus rufus*) by marking and recapturing their parasites (lice). Whereas social microbe transmission in asocial species would mainly mean sexual or agonistic contact, in social species similarity of microbiota might be a good proxy for the strength of a social bond (Montiel-Castro et al. 2013).

In addition to similarity within groups, socially transmitted gut microbes might enhance gut microbial diversity as groups get bigger. However, I found no correlation between group size and the gut microbial diversity. This is likely to be due the small variation in group size (2-6). In addition, Montiel-Castro et al. (2013) pointed out that it is not group size per se, but rather modular group organization that is associated with the benefits of socially transmitted microbes. Unlike our pair-living, cooperative study species, bigger primate groups tend have high modularity, leading to partial social isolation of different social modules within one group. The diversity of microbiota might have been one factor driving the evolution of large group size, but selective (modular) bonding...
patterns may have risen with a need to balance the transmission of beneficial microbes and pathogens (Montiel-Castro et al. 2013). Indeed, social modularity is known to reduce parasite transmission (Griffin & Nunn 2011).

Since bonding behavior in primates serves as a good way for microbial transmission, one needs to decide carefully with whom to affiliate. Affiliating with a small set of close kin or friends is known to reduce stress (Wittig et al. 2008). In addition to this, it might also reduce the risk of infection. Thus, a social bond or the associated bonding behavior in highly social species can be additionally seen as a manifestation of immunological trust. In species with allomaternal care, like red-bellied lemurs all individuals are tightly bonded and seem to share the microbiota, hence no modularity is needed.

4.4. **Gut Microbiota, HPA-axis and Individual Differences in Social Behavior**

Contrary to my prediction, both sociality indices (Huddle index and Groom index) were negatively correlated with gut microbial diversity between individuals. The negative correlation between sociality and gut microbial diversity was significant and strong before infants were born, not after they were born. Whereas gut microbial diversity decreased from before to after infants were born, individual huddle indices decreased but groom indices stayed the same. The fact that the negative trend disappears after infants are born could be explained by decreasing physiological dissimilarity between individuals at the time when infants are born. Family members most likely have more different physiological states while females are pregnant. In addition, individuals could have more similar physiological states after infants are born when all individuals might experience stress or other synchronized hormonal changes related to allomaternal care (Ziegler 2000).

Before infants were born, individuals that participated more in grooming, groomed others more and slept in close contact with others, had the lowest gut microbial diversity. This is opposite to what was predicted, assuming that grooming functions as a way of gut microbial transmission. Increased microbial transmission, however, might not always lead to higher microbial diversity.
Since the gut microbiota is a complex system with differentially transmittable microbes, social contact might only increase transmission of the plastic gut microbes belonging to the “peripheral microbiota”. This in turn could potentially lead to an increase in the relative abundances of these taxa, while decreasing the stable “core microbiota” that is not socially transmittable. Then, if the core microbiota would be much more diverse than the peripheral, increase in transmission of the latter might actually lead to decreasing diversity of the overall gut microbiota. An alternative, and probably more likely explanation for the surprising trend is that there is a third factor causing both the reduction in diversity and increase in social behavior. For example, it is plausible that both of these are caused by stress. Stress is known to reduce gut microbial diversity in mammals (Bailey & Coe 1999, Bailey et al. 2010) and is also associated with increasing grooming rates in primates (DeWaal 2000, Aureli et al. 2002, Engh et al. 2006). This would be apparent both between individuals with different HPA-axis activity and between non-stressful and stressful times. Indeed, cortisol levels are known to be high in this species during the time when infants are born (Tecot 2008).

I did not have sufficient amount of hormonal samples to test for the effect of glucocorticoids to both social behavior and gut microbiota. However, I did find fecal cortisol levels to covary with different gut microbiota, though this effect was not evident in gut microbial diversity as expected, but rather in the composition of the bacterial community. This raises the important question of whether different gut microbes in fact metabolize cortisol differently, yielding an erroneous estimate for cortisol levels from metabolites (Goymann et al. 2012). In support of this, Wohlgemuth et al. (2014) found that low gut microbial diversity was associated with abnormal steroid metabolism in mice with gut inflammation. The fact that I found no correlation between gut microbial diversity and cortisol levels might be because the hormonal data used here were limited and thus delicate associations may not have been detected.
4.5. Seasonal change in Gut Microbiota

Seasonal change in the gut microbial composition was small but significant. Both diet and weather changed greatly during the 6 months of the study, but these environmental changes had relatively little effect on the microbiota. This is somewhat surprising, because diet has been shown to have a major effect on gut microbial composition in other species (Turnbaugh et al. 2009a, Gomez et al. 2015). However, the observed decrease in diversity was accompanied by increasing abundance of bacteria belonging to genus *Prevotella*, which is linked with fibre-rich diet in humans and gorillas (Wu et al. 2011, Gomez et al. 2015) and could be a good indicator of changing diet in this species. Furthermore, the observed seasonal change in the gut microbial composition was especially prominent during the time when infants were born. Although gut microbial composition changed in all individuals at this time, I did not detect decreasing microbial diversity in the breeding females in a separate analysis. However, this might be because of the smaller sample size used. In other individual, decreasing microbial diversity was visible after controlling for the effects of pregnancy. In addition to changes in social behavior or stress physiology, the observed decrease in diversity could be a regular change associated with infant’s birth. In addition to pregnant females having modified microbiota, all individuals might experience similar seasonal modification associated with infants’ birth. For example, human mothers are known to have decreasing gut microbial diversity and richness from mid-gestation towards the end of pregnancy and this is suggested to be adaptive adjusting of microbiota to enhance beneficial maternal transmission and reduce the risk of pathogen infection from the mother to the baby (Koren et al. 2012). This process might be under hormonal control and since in species providing allomaternal care, all group-members might reflect the hormonal status of the pregnant female (Storey et al., 2000, Nunes et al., 2001, Ziegler et al., 2004, Tecot 2008), this change could happen in all individuals readying the social environment for the newborn infant.
4.6. Future directions and a framework for social transmission of beneficial gut microbiota

Social factors can be more important in determining individual gut microbiota than previously thought. In addition to early exposure, social environment continues to modify individual microbial community and microbes in turn might modify social behavior through their effects on central nervous system (Microbe-Gut-Brain axis, Montiel-Castro et al. 2013) and hormones. Since the microbiota is a key factor in immunity and social recognition, natural selection should further favor this positive loop of social microbe transmission. This said, I propose a new testable framework for the transmission of beneficial microbes in social groups and the evolution of social behavior (Fig. 10).

**Figure 9.** Framework for positive feedback loop of social transmission of beneficial gut microbes and the evolution of sociality
Not all socially transmittable microbes are beneficial, however, and an interesting future field of research lies in recognizing situations where an individual can gain beneficial microbe transmission while avoiding pathogen transmission. To test this idea, it will be important to study less social species that will exhibit the initial forms of sociality (e.g., tolerance of conspecifics, sexual pair-bonding, communal nesting). This will provide an opportunity to test whether individuals choose their social company in respect to current immunological challenges, for example by avoiding conspecifics with dissimilar microbiota and preferring the company of those with more similar microbiota. This could lead to social discrimination patterns between individuals with a different initial genetic basis for immunity (e.g. MHC-genotype) and since this might be conflicting with a good mating strategy (mating with a different MHC—genotype Hamilton & Zuk 1982, Potts et al. 1991, Apanius et al. 1997), there could be a fluctuating trade-off between current immunological benefits and the immunological quality of offspring. That is, social selection counteracting sexual selection on immunity.

Hormones form the physiological basis of both within and between-individual differences in behavioral profiles and their role as a link between microbiota and social behavior should be more experimentally studied. Specifically, the interplay and associations of oxytocin, glucocorticoids and testosterone on microbiota should be investigated, since these hormones play a key role behind behaviors such as care, affiliation, social withdrawal and territorial aggression. In addition to the interactions of microbiota and hormone levels, the effects of different gut microbiota on hormone metabolizing patterns need to be analyzed to validate the fecal hormone assays. This could be done with the HPLC-method, separating hormonal metabolites to see if different metabolites covary with abundances of different gut bacteria.
5. Conclusions

Ideas of microbes as a link between ecosystem biodiversity and human immunity have brought microbiome research “out to the wild” and launched the holobiome approach in ecology. No doubt evolutionary biology would benefit from similar revolutionary redefining of individuality. In the evolution of cooperative groups for example, the functional unit of a species shifts from individuals towards groups. Cooperation by definition requires individual to work as one, and thus it is no surprise that group-level alliances promote physiological and behavioral synchrony. In the holobiome world, however, there are no simple alliances: Evolution of social groups is refraining ecosystem by affecting connectedness between individuals. These connections in turn create whole new niches for microbial life, increasing the complexity of these socio-ecosystems. It is becoming increasingly clear that individuals, groups and species are an inseparable part of their surroundings, and populations can be seen more holistically as transmission nets for genes, cultures and microbes.

The holobiome approach aims at more comprehensive understanding of ecosystems and their dynamics. This is crucial knowledge in the fast-changing world, where more knowledge of the sensitivity of organisms to the human introduced rapid environmental chance is acutely needed. For example, we know little about the effects of human-related stress on the dynamics of social systems of highly social species. Does acute or maternal stress affect cooperative strategies? Are complex social systems sensitizing or buffering species towards environmental change? Another example is the emerging understanding we have of the effects of local biodiversity on the development of autoimmune diseases in humans (Hanski et al. 2012, Heederik et al. 2012). This kind of research requires multi-disciplinary cooperation between different fields of biology and other natural sciences. In this way, the holobiome approach has great potential in bringing together thoughts and ideas, separated by the reductionist philosophy of science in the last century. Just like social interaction might increase microbial transmission and immunity, scientific interaction will increase transmission of thoughts and the potential we have for solving the problems facing us.
6. Acknowledgements

This thesis is done in the Metapopulation Research Centre at the University of Helsinki, under the supervision of professor Ilkka Hanski, Dr. Lasse Ruokolainen, Dr. Stacey Tecot (University of Arizona) and Dr. Andrea Baden (Hunter College, City University of New York) in collaboration with Dr. Katherine Amato (University of Colorado). I would like to thank all my supervisors for this opportunity and for precious support, critique, advice and trust. Special thanks also to Dr. Katherine Amato for endless help and the best possible introduction to microbial DNA-sequencing methods.

I am also forever grateful to Avery Lane for the groundbreaking scientific ideas behind this project. Field work carried out by me and Avery Lane was made possible by permission of MNP and permits provided by MICET-office, our expert field technicians Pierre and Dominique and Centre ValBio research station in Ranomafana, Madagascar. I also want to thank the many funders of this project: University of Helsinki and Jane & Aatos Erkko Foundation for funding my work, Stacey Tecot and Andrea Baden for funding the field work, University of Arizona, Rowe-Wright Primate Fund and American Association of Physical Anthropologists for funding the Red-Bellied-Lemur Allomaternal Care project, of which side-project my thesis was, and Earth Microbiome Project for funding the microbial DNA-analyses.

Lastly, I would like to give special thanks Dr. Sarah Zohdy, who inspired me to seize these ideas, Wolfgang Goymann for sincere advice and Matan Shenhav, Ilona Jetsonen, and Ferhat Kaya for most insightful and inspiring conversations.

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