Activity budget and gut microbiota across reproductive states in wild, female capuchin monkeys in a seasonal dry forest

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ABSTRACT

Energy demands associated with pregnancy and lactation are significant forces in mammalian evolution. To mitigate increased energy costs associated with reproduction, female mammals have evolved behavioral and physiological responses. Some species alter activity to conserve energy during pregnancy and lactation, while others experience changes in metabolism and fat deposition. Restructuring of gut microbiota with shifting reproductive states may also help females increase energy harvest from foods, especially during pregnancy. Here, we combine life history data with >13,000 behavioral scans and >300 fecal samples collected longitudinally across multiple years from 33 white-faced capuchin monkey females to examine the relationships among behavior, gut microbiota composition, and reproductive state. We used 16S-based amplicon sequencing and the DADA2 pipeline to analyze microbial diversity and putative functions. Reproductive state explained some variation in activity, but overall resting and foraging behaviours were relatively stable across the reproductive cycle. We found evidence for increases in biotin synthesis pathways among microbes in lactating females, and that microbial community dissimilarity among the states was small but significant. Otherwise, gut microbiota structure and estimated functions were not substantially different among reproductive states.

These data contribute to a broader understanding of plasticity in response to physiological shifts associated with mammalian reproduction.

INTRODUCTION

The demands of pregnancy and lactation have been an influential force throughout mammalian evolution. Female mammals experience discrete stages of the reproductive cycle, including cycling, pregnancy, and lactation, but variation across mammalian taxa exists in response to cycling parameters, litter size, birth weight, gestation length, weaning age, weaning mass, and interbirth interval (Gittleman & Thompson, 1988). Lactation is typically the most energetically demanding stage of the reproductive cycle because milk production and other aspects of infant care, incuding infant carrying, require considerable energy above basal metabolic function (Clutton-Brock et al., 1989; Dewey, 1997; Gittleman & Thompson, 1988). Pregnancy is the second most energetically-demanding state, and non-pregnant, non-lactation states (i.e. cycling and non-cycling pauses) are the least energetically costly (Dufour & Sauther, 2002; Serio-Silva et al., 1999). In addition to heightened net energy demands, pregnancy and lactation also introduce increased protein and other nutrient requirements to fuel fetal and infant growth (Dewey, 1997; National Research Council, 2003). Energy requirements typically increase as a fetus develops during pregnancy; after parturition, energy demands continue to increase as the mother produces milk (Ellison, 2003; Emery Thompson, 2013; Villar et al., 1992). As the infant grows and needs more milk combined with larger infant size, energy demand on the mother continues to grow. During the final stages of lactation, once the infant becomes semi-independent in the lead-up to weaning, energy requirements related to infant care decrease (Figure 1).

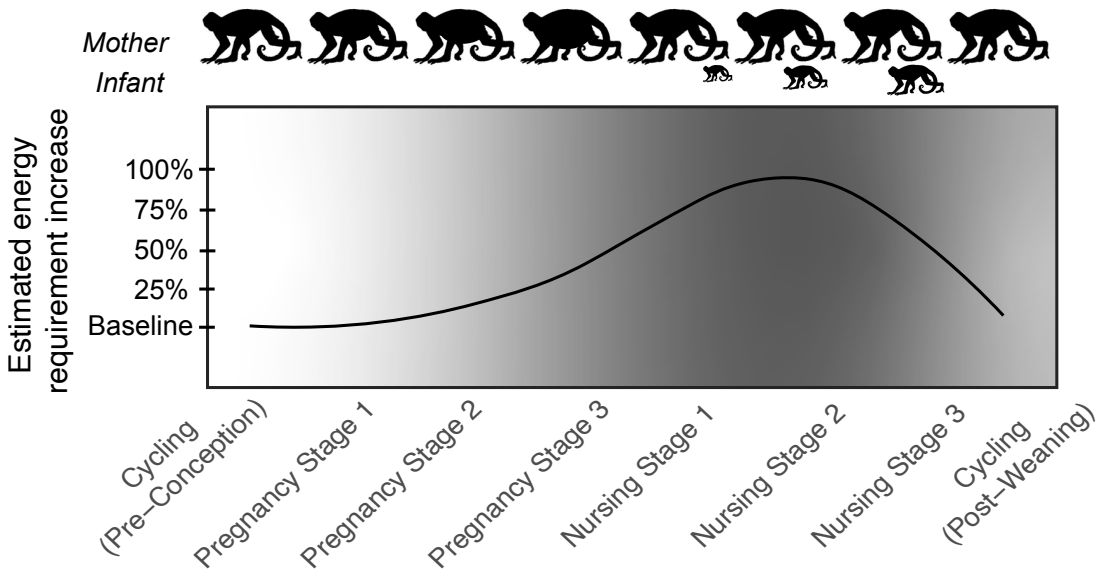

Figure 1. Visualization of estimated increases in energy requirements during the reproductive cycle of a non-human primate. Female primates face a 25% increase in daily energy requirements during gestation, and up to a 50-100% increase during lactation (Key & Ross, 1999).

Mammals vary widely in the length and energy costs of reproduction and have evolved multiple strategies in response. Adaptations include behavioral responses to seasonal fluctuations in food availability. For example, harbour seals (*Phoca vitulina*) and other migratory species travel to specific breeding sites and feeding sites, and exhibit strictly seasonal breeding that is tied to food abundance in their environment (Bowen et al., 2001). For mammals that are not constrained by migratory patterns or strict seasonal breeding, behavioral flexibility—including modulating energy expenditure, foraging rates, and food choice—offers a strategy to mitigate increased energy costs of pregnancy and lactation. Primates, including humans, exemplify these behvioral strategies. While some primates conserve energy during costly reproductive stages by resting for larger proportions of the day (e.g. red-ruffed lemurs [*Varecia rubra*], (Vasey, 2005); green monkeys [C*ercopithecus sabaeus*] (Harrison, 1983)), others increase energy intake, either by foraging for longer periods of the day (e.g. yellow baboons (*Papio cynocephalus*) (Altmann,

2001)) or by increasing their intake rate of foods (e.g. white-faced capuchins [*Cebus capucinus imitator*] (McCabe & Fedigan, 2007)).

Adaptations to the demands of the reproductive cycle also include physiological changes that occur within the mother. For example, changing patterns of fat gain enable females to accumulate fat stores during pregnancy that they can draw from during lactation. Humans typically experience increased fat deposition during pregnancy, even in cases when they are experiencing food stress (Poppitt et al., 1993). Similar results were found in a study of captive bonobos (*Pan paniscus*), in which pregnant females did not lose weight while experiencing caloric restriction (Deschner et al., 2008). Sufficient temporary fat gain during pregnancy supports efficient and healthy development of infants. Too little fat gain may lead to increased periods of lactation and increased interbirth interval (e.g. humans (Lunn et al., 1984)), while too much fat gain during gestation can lead to birth complications (e.g. domestic canines and felines (Fontaine, 2012)).

Research on humans suggests that pregnancy is also associated with changes in gut microbial communities (DiGiulio et al., 2015; Koren et al., 2012; Smid et al., 2018). These changes, which include reduced diversity of microbes, shifts in prominent bacterial phyla associated with energy harvest, and shifts in putative metabolic pathways related to energy absorption are linked to metabolic disease states in non-pregnant individuals. However, in the context of reproductive demands, they may serve an adaptive role in increasing energy harvest from food during times of increased energy need for fetal development and allow for increased fat storage to cope with costs of lactation (Edwards et al., 2017; Koren et al., 2012). In non-human mammals, evidence suggests gut microbiota change during reproduction (e.g. Tibetan macaques (Sun et al., 2021)), and shifts may be hormonally mediated (Mallott et al., 2020). However, other researchers have found that composition and predicted function of individuals' gut microbiota remained relatively static throughout pregnancy and into early lactation (Jost et al., 2013). These contrasting findings may be due to differences in study design, methods, and sample species and population. Alternatively, they may indicate that the degree to which the gut microbiome can shift during pregnancy is constrained or enabled by external factors.

Behavioral and gut microbial changes might interact to address the demands of pregnancy and lactation. However, few studies have combined behavioral and gut microbial data tracked throughout pregnancy and lactation to understand the nuances of how mammals in a wild setting cope with increased energy requirements. Here, we combine behavioral and gut microbial data from a well-studied population of wild non-human primates to examine the strategy or combination of strategies that female primates employ to address the increased energetic costs of pregnancy and lactation. To examine behavioral and gut microbial community flexibility as they relate to the reproductive cycle, we studied a population of omnivorous, wild white-faced capuchin monkeys that exhibit moderately seasonal breeding. Specifically, we examine white-faced capuchin monkey responses to changing reproductive stages over the course of 5 years in a seasonal dry forest. We combine a robust data set of >13000 behavioral scans with >300 fecal samples collected from 33 monkeys to study behavioral and gut microbial responses to reproduction in a species that inhabits a dynamic and seasonal ecosystem. Our first aim was to compare activity budgets of white-faced capuchins among and within cycling, pregnancy, and nursing stages. We predict that if capuchins employ an "energy conservation" approach during pregnancy and nursing, then females will rest more in stages of higher energy demand compared to stages of lower energy demand. Conversely, if capuchins employ an "energy maximization" approach during pregnancy and lactation, then females will forage for larger proportions of their day compared to cycling capuchins. Our second aim was to investigate gut microbial changes in female capuchins among cycling, pregnant, and nursing states. We predict that gut microbiota will exhibit characteristics associated with increased capacity for energy harvest during periods of highest energy demand during pregnancy. We also predict that females' gut microbiota will exhibit an increase in relative abundance of putative metabolic pathways related to energy metabolism and carbohydrate transport during pregnancy. Given the demonstrated potential for ecological and social factors to influence behavioral or gut microbial flexibility in this species, we additionally examine the potential effects of precipitation, temperature, diet, fruit biomass in the environment, and dominance rank on activity budget and gut microbial communities.

RESULTS

Aim 1: Compare activity budgets of white-faced capuchins among and within cycling, pregnancy, and nursing stages

To visualize overall activity budget shifts across the reproductive cycle, we combined related behaviors (see Ethogram, Supplemental Table 1) into six general categories: Foraging, Resting, Social Affiliation, Social Aggression, Travel, and Other. We calculated proportions of each category per total scans per day (Figure 2).

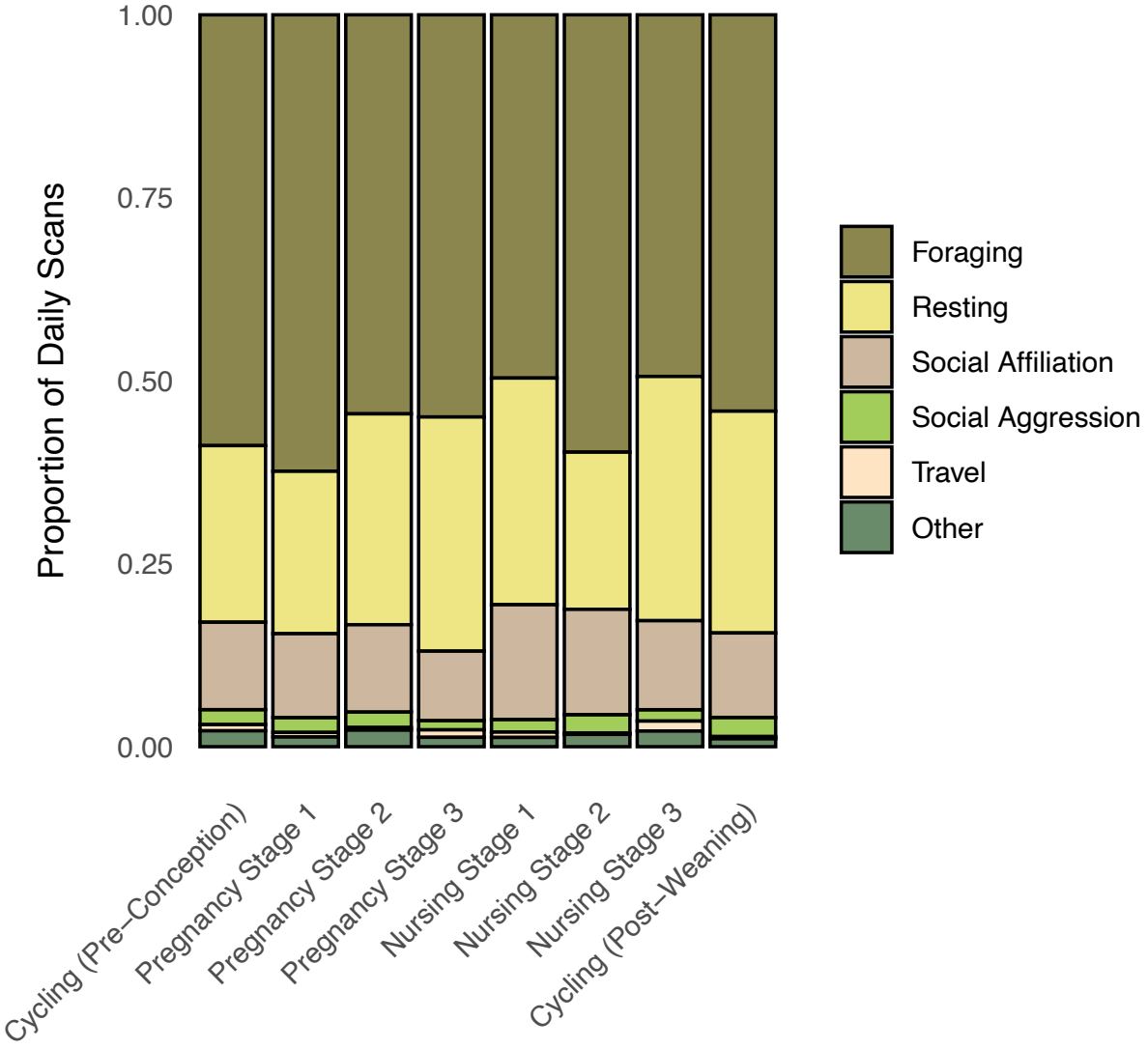

Figure 2. Proportions of daily scans spent in each of six behavioral categories across the reproductive cycle. These data represent raw counts of scans per behavior per day divided by total scans recorded per day.

Resting activity within and among reproductive states

A generalized linear mixed model of resting activity that included reproductive state outperformed a null model excluding this variable, suggesting some variation in resting behavior was explained by reproductive stage. High social rank was significantly negatively related to total resting scans (Estimate = -0.13, SE = 0.06, Z-Value = -2.16, $p = 0.03$), indicating that higher ranking individuals rested less often than lower or mid-ranking individuals. Maximum temperature was significantly positively related to total resting scans indicating that monkeys rested more often in hot temperatures (Estimate = 0.22, SE = 0.02, Z-Value = 10.35, $p < 0.001$) (Supplemental Table 2). Incident Rate Ratios for all predictors are presented in Figure 3a and values reported in Supplemental Table 2. Predicted counts of resting scans per day are visualized in Figure 3b and demonstrate that resting increased throughout pregnancy and early nursing, dipped in mid-nursing, and increased again in late nursing. However, variation was minor and we did not find significant pairwise differences among the eight individual reproductive stages.

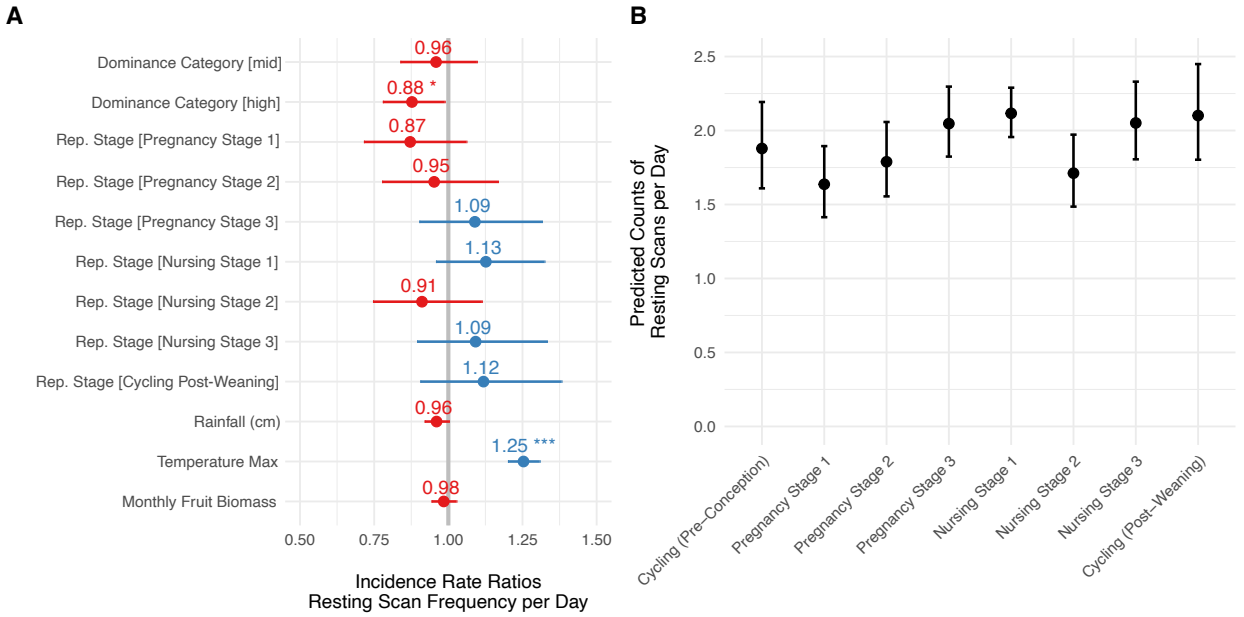

Figure 3. Incidence rate ratios (IRR) and standard error (**A**) for predictors from a GLMM of resting scans per day. The reference dominance category is low social rank; the reference reproductive stage is cycling (pre-conception). The grey vertical line represents "no effect". Values to the right of the grey line represent positive effects and values to the left represent negative effects. Significant predictors ($p < 0.05$) are denoted with asterisks. We present an alternative way to visualise the effect of reproductive stage on resting activity by plotting the predicted number of resting scans per day for each level of reproductive stage variable (**B**).

Foraging activity within and among reproductive states

The generalized linear mixed model of foraging activity that included reproductive state outperformed a null model excluding this variable. Females in Nursing Stage 1 exhibited significantly fewer foraging scans per day compared to other stages (Estimate = -0.13, SE = 0.05, Z-Value = -2.45, p = 0.01). Ecological variables including rainfall, daily maximum temperature, and estimates fruit biomass were also significantly correlated with foraging scans per day and values are reported in Supplemental Table 2). Incident Rate Ratios for all predictors in the model are presented in Figure 4a and we visualized predicted counts of foraging scans per day in Figure 4b. These predicted counts, which take into account all other predictors in the foraging model, suggest that foraging scans steadily decreased throughout pregnancy and into early nursing before increasing throughout late nursing and into post-weaning cycling.

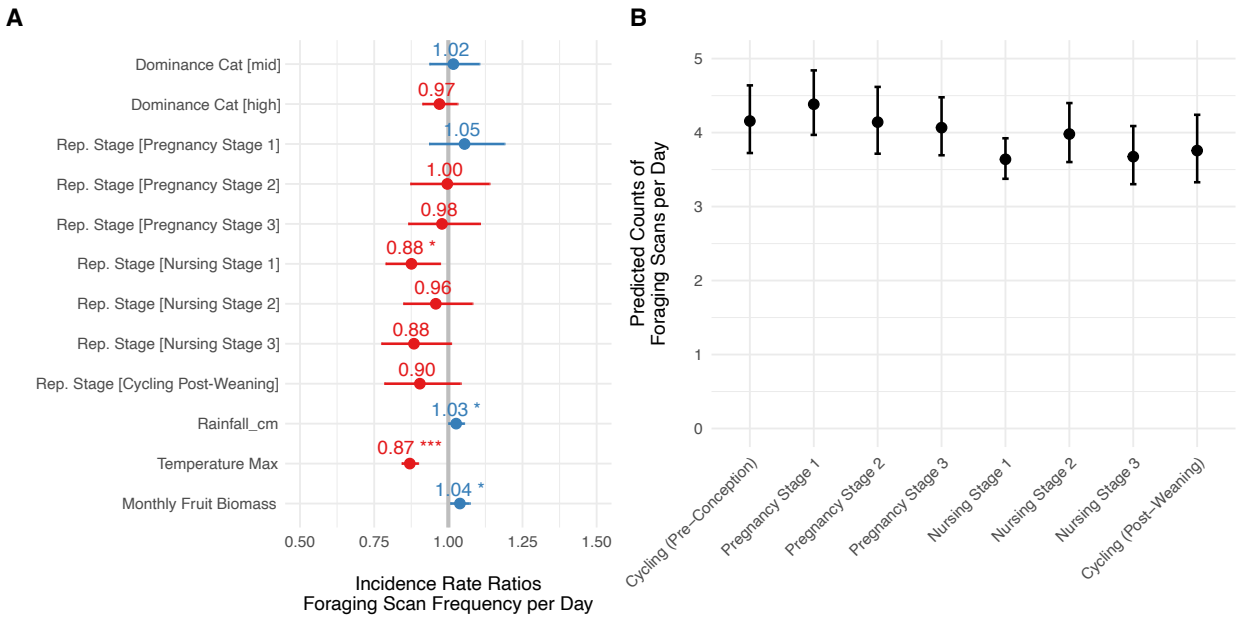

Figure 4. Incidence rate ratios (IRR) and standard error (**A**) for predictors from GLMM of foraging scans per day. The reference dominance category is low social rank; the reference reproductive stage is cycling (pre-conception). The grey vertical line represents "no effect". Values to the right of the grey line represent positive effects and values to the left represent negative effects. Significant predictors ($p < 0.05$) are denoted with asterisks. We present an alternative way to visualise the effect of reproductive stage on foraging activity by plotting the predicted number of resting scans per day for each level of reproductive stage variable (**B**).

***Aim II:* Investigate gut microbial changes in female capuchins among cycling, pregnant, and nursing states**

Gut microbial community structure remained consistent among reproductive states

Overall, when we examined broad metrics of gut microbial community structure, we found that reproductive state was not a significant predictor of the Chao1 species richness or Shannon alpha diversity (Supplemental Table 3). Rainfall was significantly negatively correlated with Chao1 richness (Incidence Rate Ratio = 0.89, CI = 0.83 – 0.95, p = 0.001), but no other predictors were significant in either model. Reproductive status was not a significant predictor of gut microbial community dissimilarity (DF= 2, F = 1.275, R^2 = 0.008, P = 0.163) and samples from the same

200 reproductive state did not cluster distinctly (Figure 5). Individual identity accounted for a
201 statistically significant degree of dissimililarity among samples (DF = 28, F = 1.278, R^2 = 0.11, P
202 = 0.003), as did diet type and rainfall (Supplemental Table 3).
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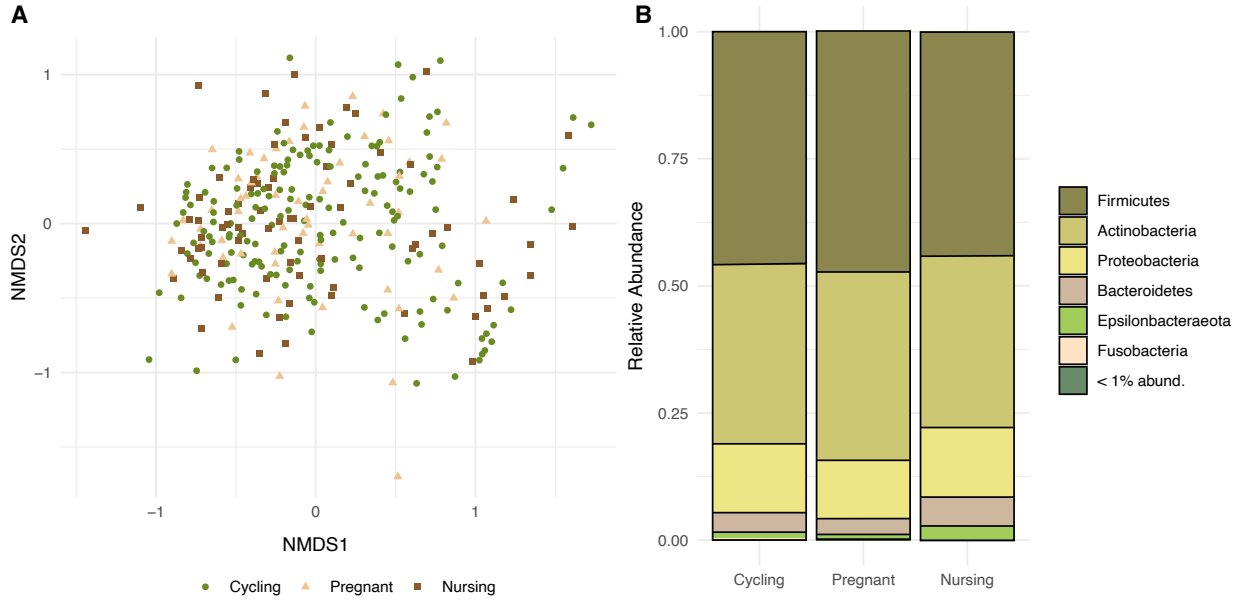

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205 **Figure 5**. For each sample, Bray-Curtis dissimilarity values were computed and ordinated using
206 non-metric multidimensional scaling (NMDS). Samples did not cluster according to reproductive
207 status which was also not a significant predictor of dissimilarity among fecal microbial
208 communities. Relative abundance of phyla were visualized across reproductive statuses. Phyla
209 with relative abundances below 0.01 were grouped in the category "<1% abund." Relative
210 abundances remained generally stable across the reproductive states.
211

212 To investigate other structural changes in the fecal microbial communities among reproductive
213 states, we visualized the relative abundance of phyla across the reproductive cycle, grouping all
214 phyla with relative abundances lower than 1% (Figure 5b). We found that pregnant females
215 exhibited a small but significant increase in Firmicutes taxa compared to cycling females (log2
216 fold change = 0.493, SE = 0.146, P = 0.008). Cycling females exhibited a small but significant
217 increase in the phylum Epsilonbacteraeota (log2 fold change = 1.149, SE = 0.293, P < 0.001).
218 We also examined whether specific genera were differentially abundant among the reproductive
219 states and found that cycling females exhibited a significant increase in taxa from the genus
220 *Helicobacter* compared to nursing females (log2 fold change = 1.226, SE = 0.309, P = 0.01).

Estimated metabolic pathways remain largely stable among reproductive states

Reproductive status was a significant predictor of estimated metabolic pathway dissimilarity, but the effect was small (PERMANOVA; df =2, F Value = 2.5075, $R^2 = 0.017$, $p < 0.001$). Nursing females were characterized by a significant increase in pathways related to biotin metabolism (Linear Discriminant Analysis; LDA Score = 3.176, p = 0.006), but otherwise metabolic pathways did not differ substantially between reproductive statuses.

DISCUSSION

We analyzed a robust set of >13,000 individual scans to explore behavioral responses to reproduction and 308 fecal samples to understand gut microbial community dynamics in population of reproductively mature female capuchin monkeys. Our main findings are 1) reproductive state explains some variation in activity budget; in particular, foraging decreases significantly in early nursing compared to cycling, though resting and foraging activity remain otherwise stable across the reproductive cycle; 2) reproductive state explains some variation in gut microbial dissimilarity and relative abundance of putative metabolic pathways; 3) ecological and social variables including maximum temperature, social dominance, and estimated fruit biomass, as well as individual identity are related to activity and the gut microbiota more strongly than reproductive status.

Activity budgets remain largely consistent within and among reproductive states

In our first aim we sought to compare activity budgets of white-faced capuchins among and within cycling, pregnancy, and nursing states at a fine scale. Resting behavior was largely consistent across the reproductive cycle, but the variation observed suggests that resting increases slightly during pregnancy. This pattern fits well with expectations from available data on energy expenditure. While no single stage in the reproductive cycle was stastically significantly different from the other stages in terms of resting, we observed a general pattern that suggests resting increases during pregnancy. Foraging behavior decreased steadily during pregnancy, possibly in connection to resting increases.

During nursing, resting behavior peaked in early nursing, before decreasing in mid-nursing, and rebounding in late nursing. Foraging behavior decreased steadily into early nursing, where it was significantly lower in early nursing compared to other stages. We recognize that proportion of scans per day spent in foraging states is an imperfect estimator for amount of food consumed. Nevertheless, the statistically significant drop in foraging behavior during early nursing may have energy balance implications for females. Capuchins may alter behavior in other ways to cope with changing energy needs. For example, past research on a small subset of the current study population suggests that lactating females increase feeding rate (McCabe & Fedigan, 2007), though we unfortunately lack the required depth of focal data to test this hypothesis in the current data set. There might also be underlying metabolic or other physiological changes such as metabolic shifts associated with energy sparing that we were not able to capture in the present study that help pregnant and lactating females address energy costs. Even though our sample size of 33 females tracked over multiple years and pregnancies is one of the largest available for wild primates, the pattern we observed in the resting behavior may be too subtle to reach significance with present sample sizes.

While capuchins are generally considered highly flexible and plastic in response to changes in their environments (Fragaszy et al., 2004), it is likely that both resting and foraging behavior is relatively constrained in this population and are influenced by social and environmental factors, limiting the potential for flexibility in this domain in response to reproductive state. When food and water resources change drastically from season to season, capuchins alter their foraging and ranging behaviors to overlap with available food and water (Campos & Fedigan, 2009). We also see changes in thermoregulatory behaviors; capuchins rest more during the hottest parts of the day in the hotter season of the year, and exhibit seasonal behaviors likely linked to thermoregulation and water consumption. It is also possible that female capuchins are constrained in altering activity budget due to the pressures associated with group living. White-faced capuchins form cohesive groups, with the exception of emigrant males dispersing to non-natal groups. Females remain the same social group their entire lives (with the rare exception of group fissioning events), and capuchins forage, rest, and travel in close proximity to one another. Pregnant and lactating females may theoretically benefit from resting for longer periods of the

day or foraging for longer periods in a particularly productive food patch; however, if a pregnant or lactating female acts independently of the larger social group, she may be increasing risk of predation or encounters with other social groups. The ability of females to significantly alter resting or foraging may be constrained by the behavioral choices in the rest of the social group. Future studies examining these constraints on activity budget shifts represent an exciting future avenue of behavioral research.

Gut microbial communities remain largely stable structurally and functionally throughout reproduction

We found mixed support for the prediction that females modulate the gut microbiome to increase energy absorption from food during pregnancy and nursing. We did not observe statistically significant changes in alpha diversity in pregnant females, which contrast previous studies in humans that showed a drastic decrease in alpha diversity (Koren et al., 2012). Females in cycling, pregnant, and nursing states clustered separately in a beta diversity plot, but the effect was very small, suggesting alternative drivers of community dissimilarity, including individual variation. Overall, female capuchins did not exhibit large gut microbial structural shifts, but we did find that pregnant females exhibited small but significant increases in Firmicutes taxa. At a broad scale, taxa within Firmicutes break down carbohydrates that endogenous host enzymes are unable to metabolize. The ratio of Firmicutes to Bacteroidetes has previously been suggested as a biomarker for increased metabolic activity in the gut (Turnbaugh et al., 2006); however, contrasting reports of this ratio suggest it might not serve as a universal biomarker for increased energy (Magne et al., 2020). Further, the lack of substantial change in relative abundance among bacterial genera in our samples suggest that, in this population, reproductive state is not a critical driver of gut microbial community composition, at least at a broad scale.

Though gut microbial communities remained largely stable, nursing capuchins in our population exhibited a significant increase in a biotin metabolism pathway in the gut. Endogenous enzymes as well as gut microbes can metabolize biotin, which is involved in a broad range of metabolic processes related to fat-, carbohydrate-, and amino acid-utilization in mammals. Biotin deficiency and biotin excess during the reproductive has been linked to tetratogenic effects in

pregnancy in mice and humans (Báez-Saldaña et al., 2009). Studies of humans have repeatedly demonstrated that lactation and pregnancy alter biomarkers of biotin metabolism, and that humans are typically deficient in biotin during pregnancy, though precise requirements of biotin remain unknown (Mock et al., 2002; Perry et al., 2014). If non-human primates also require increased biotin during gestation and lactation, our results suggest that the gut microbiome may play an important role in helping nursing females increase biotin supplementation during fetal growth and infant development.

Previous studies of non-human primates tend to suggest that the gut microbiome shifts considerably throughout the reproductive cycle (Amato et al., 2014; Mallott et al., 2020; Mallott & Amato, 2018). In a recent examination of white-faced capuchin reproductive microbial ecology, Mallott and Amato (2018) examined how gut microbial communities changed across reproductive states in females ($n_{females} = 5$, $n_{samples} = 39$) sampled across one year in an aseasonally breeding population of white-faced capuchins. The authors found evidence to suggest that the gut microbiome shifts significantly during the reproductive cycle, including differences in relative abundance of Firmicutes (lower in pregnant versus cycling females) and Actinobacteria (higher in pregnant versus lactating females). Further, the authors found that reproductive state was significantly associated with energy and glycan metabolism (Mallott & Amato, 2018). However, this capuchin population lives in a wet aseasonal forest, with little variation in food and water availability throughout the annual cycle (Mallott et al., 2018). The biome where our present study took place is, by contrast, highly seasonal, with distinct shifts in temperature, water availability and fruit and arthropod abundance (Campos et al., 2015; Mosdossy et al., 2015). In the hot, dry season animals contend with harsh drought and high temperature, while in the rainier, cooler season, these forces are less present. We have observed strong effects of seasonality on ranging behavior, activity budget, food choice, and the gut microbiota in this population (Campos et al., 2014; Campos & Fedigan, 2009; Melin et al., 2020; Orkin et al., 2019; Orkin et al., 2019). Extreme seasonality at the present study site and aseasonality at a different site that is home to the same species of capuchins may have critical implications for our understanding of how flexible and plastic this species is across its home range.

Capuchins are generally thought of as one of the most flexible and generalist species of platyrrhine primates that can thrive in a wide variety of habitats and that can consume a vast diversity of food types (Fragaszy et al., 2004; Melin et al., 2020). However, the reality might be a bit more nuanced. Steig Johnson and Kerry Brown (2018) examined niche breadth in Mesoamerican primates using an ecological niche modeling approach, and found that capuchins were considerably constrained by several ecological factors, including precipitation in particular seasons, and seaonality of temperatures. The temperatures and water availability at our study site near the limit of suitable conditions for this species, which may explain the lack of flexibility that we see in behavior and gut microbiota in this population. Understanding how flexibility shifts across a species range and identifying what ecological factors permit or constrain a species' ability to be flexible, is critical to understand not only that species' history, but also how it might fare as ecosystems face anthropogenic and climate-related changes.

Alternatively, we may be missing the importance of individual variation in response to reproductive states. For example, humans residing in the same population display remarkable differences in response to reproductive demands across our global range; for example, women in the Gambia and Sweden experience high within-group variation in weight gain and energy expenditure throughout pregnancy (Poppitt et al., 1993, 1994) and high inter-individual gut microbiota among members of the same population has been found in humans (Healey et al., 2017; Zhu et al., 2015). We found that individual identity accounted for a significant amount of gut microbial community dissimilarity, which raises exciting questions about individual strategies for coping with reproduction. Further, while activity budgets and amplicon sequencing provide important, thought relatively coarse, data about behavior and gut microbiota respectively, future research on this population of capuchin could incorporate individual focal data and/or shotgun metagenomic sequencing, both of which would provide a more detailed understanding of capuchin reproductive behavioral and microbial ecology.

How animals respond to the demands of reproduction has important consequences for the viability of offspring, and on a longer term scale, the fitness of a population or species. The intricacies of how animals are able to shift their behavior and how their gut microbial communities may respond to pregnancy and lactation represent a complex but critical area of

research. For populations living near the ecological limits of their species ranges, it is especially important to understand the extent to which plasticity in behavior and gut microbial communities might influence pregnancy outcomes and multi-generational fitness.

MATERIALS AND METHODS

Field site & study population

We collected samples and behavioral data at Sector Santa Rosa (SSR), located in the Área de Conservación Guanacaste (ACG), in Guanacaste, Costa Rica (10°53´01´´N 85°46´30´´W). Sector Santa Rosa is a mosaic of forest types, including tropical dry forest and small patches of older growth evergreen forest. The ACG experiences two distinct seasons: a hot, dry period from late November to mid-May and a cooler, rainy period for the remainder of the year, during which almost all of the annual rainfall (900 mm-2400 mm) occurs (Melin et al., 2020). Fruit abundance varies throughout the year and estimates of fruit biomass are calculated monthly (Campos et al., 2015; Orkin et al., 2019).

The study population of white-faced capuchin monkeys has been continuously monitored non-invasively since 1983. Female capuchins are philopatric and reach reproductive maturity by 6 years of age. Births are moderately seasonal at Sector Santa Rosa, with 44% of births occurring between May and July each year (Carnegie et al., 2011). Gestation is 157 +/- 8 days and typical inter-birth intervals are 2.5 years (Melin et al., 2020). Lactation lasts for approximately 12 months; in early lactation, infants are almost exclusively dependent on their mothers and are observed nursing frequently (Fragaszy et al., 2004). It should be noted that in other white-faced capuchin populations, the lactation phase can extend to 23 months (Melin et al., 2020). After the first three months of nursing, infants gradually learn to forage independently and incorporate non-milk foods into their diet. By 12 months of age, infants are rarely observed nursing or traveling dorsally on their mothers. Infant capuchins are occasionally observed nursing from adult females that are not their mothers (Sargeant et al., 2016).

406 We collected data across 5 years (2014-2018). Non-invasive fecal samples were collected from
407 33 adult females from 4 social groups during multiple sampling bouts in 2014-2016. We
408 collected behavioral data from 33 adult females from 4 social groups during multiple sampling
409 bouts in 2016-2018. One female from the 2014-2016 fecal sampling periods disappeared from
410 the population and was not included in behavioral analysis but was included in gut microbiome
411 analysis. Two females reached sexual maturity during the 2016-2018 period and were included
412 in behavioral analysis but not in gut microbiome analysis. All animals in the study population are
413 habituated to researcher presence and individually identifiable through physical markings on the
414 face and body. In our dataset, 2016 was the only year in which we collected behavioral records
415 and fecal samples simultaneously. Ideally, we would have behavioral records and fecal samples
416 for all 5 years, but this was not possible due to field and laboratory limitations. However, the
417 nearly all individuals (31/33) were present across the entire study period and each dataset
418 (behavioral and fecal) is robust and overlapping.
419
420 During bi-monthly censuses at the field site, reproductive state of each female is recorded.
421 Pregnancies during the study period were determined via protrusion of the abdomen (visible
422 approximately 8 weeks after conception), and after infant were born we estimated conception
423 dates using 157 days as gestation length. At 15 time points throughout the 5-year study, females
424 exhibited protruding abdomens consistent with pregnancy, but then were later observed with flat
425 abdomens. We characterised these instances as pregnancy lsos, though we do not have hormonal
426 data to confirm these pregnancies, which is a limitation associated with this assumption. We
427 determined nursing on an *ad libitum* basis through observations of young monkeys suckling from
428 adult females. Following Bergstrom (2015), we considered females nursing their own infants
429 <12 months of age to be lactating. Juvenile capuchins are occasionally observed suckling after
430 12 months of age, but it is difficult to determine whether milk is transferred. We did not consider
431 cases in which juveniles >12 months were suckling to be indicative of lactation in the adult
432 females from whom they were attempting to nurse. We considered females that were never
433 observed as nursing or pregnant on data collection days as non-pregnant, non-nursing. We
434 grouped all non-pregnant, non-nursing females into the category "cycling" following Bergstrom
435 (Bergstrom, 2015).
436

Studies of humans and non-human primates suggest that energy requirements change throughout pregnancy and lactation (Emery Thompson, 2013). To examine differences that occur *within* each reproductive state, we subset the reproductive states into stages: Cycling (Pre-conception), Pregnancy Stage 1 (early), Pregnancy Stage 2 (mid), Pregnancy Stage 3 (late), Nursing Stage 1 (early), Nursing Stage 2 (mid), Nursing Stage 3 (late), and Cycling (Post-weaning) (Table 1).

Table 1. Pregnancy and nursing were divided into three equal stages. Cycling (Pre-conception) consisted of 60 days prior to a conceptive event, and Cycling (Post-weaning) consisted of 60 days post-weaning.

Reproductive State	Stage	Length
Cycling (Pre-conception)	--	60-0 days before conception
Pregnancy	early	0-53 days post conception
	mid	54-104 days post conception
	late	105-158 days post conception
Nursing	early	0-121 days postpartum
	mid	122-242 days postpartum
	late	243-365 days postpartum
Cycling (Post-weaning)	--	0-60 days post weaning

The reproductive state of each of the 33 adult female capuchins is presented in Figure 6. Throughout the 2014-2018 study period, 43 infants were born in the study population. Behavioral data collection periods (2016-2018) included portions of or the full duration of 40 of these pregnancies. Of these 40 infants, 26 infants survived to weaning (365 days), and behavioral data collection included portions of all 40 nursing periods and captured transitions from nursing to non-nursing states.

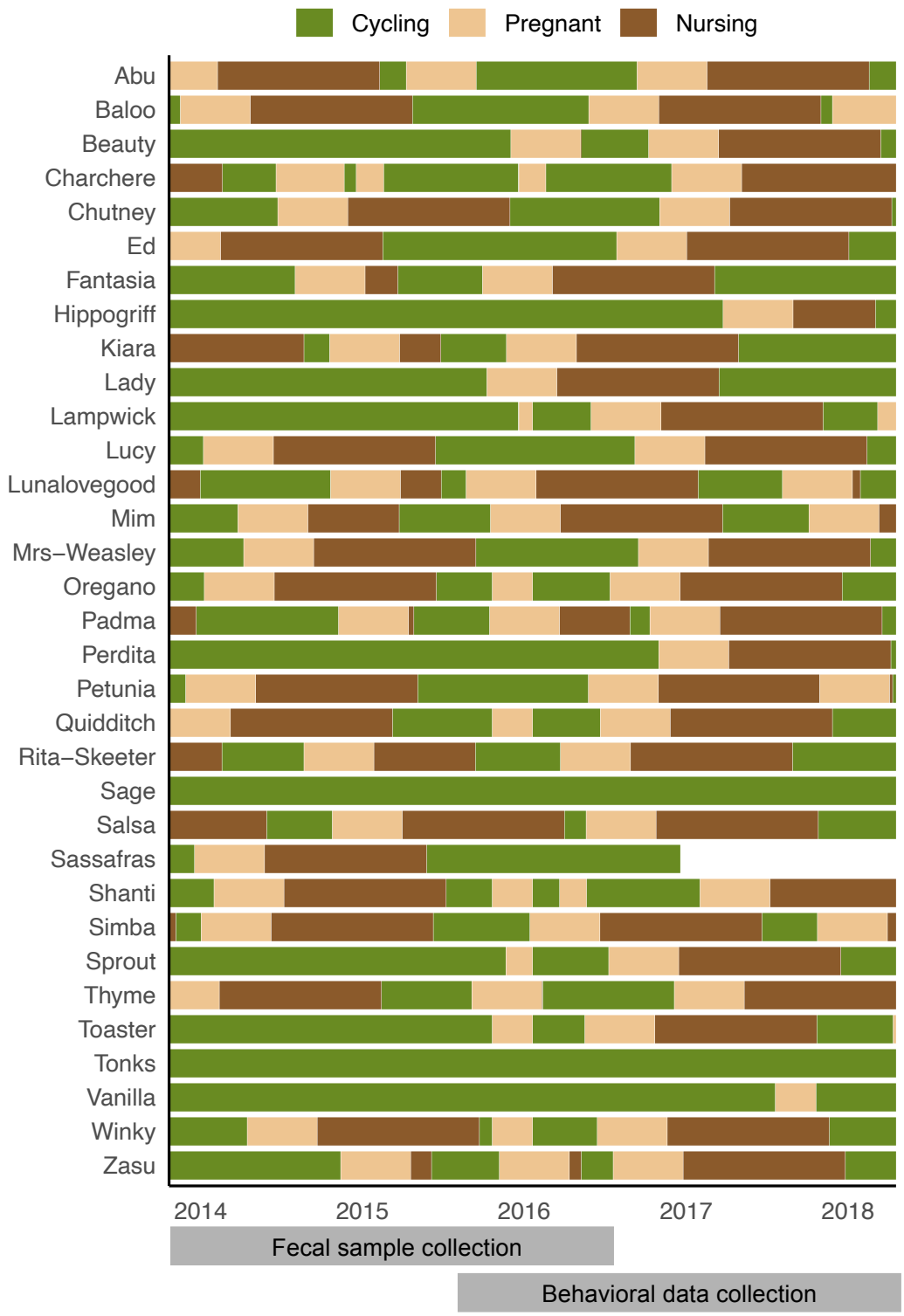

Figure 6. Reproductive status of study individuals observed between April 1, 2014 and June 30, 2018. Fecal samples were collected between April 29, 2014 and September 27, 2016. Behavioral

data were collected between April 20, 2016 and June 22, 2018. One individual (Sassafras) disappeared from the population in early 2017. Two individuals (Tonks and Vanilla) reached reproductive maturity during the study, but were never observed to be pregnant. Multiple females were observed to be pregnant via protrusion of the abdomen, but were subsequently observed with no protrusion. In these cases the tan (pregnancy) segments are followed by green (cycling) and not by brown (nursing) segments. Nursing segments that are shorter than 12 months represent cases where infants died.

Daily individual activity budgets

To determine daily activity budgets, we followed each of the 4 social groups from dawn (05:30) until dusk (18:00) for 4-6 days per month. Individual scans were recorded every 30 minutes on the hour and half hour. During a 10-minute period, we recorded the behavioral state of each individual in the group using an established ethogram (Supplemental Table 1). We chose to use scan sampling instead of focal sampling to determine individual activity budgets because it allows for more evenly distributed data across all individuals, season, and time of day (De Ruiter, 1986; Melin et al., 2018). Inter-observer reliability was tested daily for the first 4 weeks of each sampling period, then weekly or biweekly for the remainder of each period. We collected 13721 individual scans over the course of 222 contact days.

Behavioral models for activity budget analysis

We fit two generalized linear mixed models (GLMMs) that included reproductive stage as our predictor of interest. For our Resting Model, number of resting scans per day was our response variable, while for our Foraging Model, number of foraging scans per day was our response variable. In each model, we included monkey dominance category, daily maximum temperature (°C), daily rainfall (cm), and mean monthly estimated fruit biomass (kg/ha) as predictor variables as they may influence activity in this population. Ecological variables (e.g. maximum temperature, rainfall, and fruit biomass) were *z*-transformed (i.e. scaled so that each had a mean of 0 and standard deviation of 1) to stabilize the models. We included individual animal identity as a random effect in all models. Sampling effort (i.e. number of scans per animal per rotation)

489 varied due to changing field conditions and stochastic movement and dispersal of group
490 members. We included a log-transformed offset of total scans per animal per day to account for
491 differences in sampling effort. Because our behavioral data are count data and because
492 behavioral scans occur independently, a Poisson distribution with a logit link was designated in
493 all models.
494
495 We tested whether our alternative models (fixed and random effects) outperformed the null
496 models (random effects only). Likelihood ratio tests were conducted using the R function
497 ANOVA. To test for multicollinearity between ecological variables a generalized linear model
498 (GLM) was created to determine the variance inflation factor (VIF) (Craney & Surles, 2002).
499 These models were identical to the alternative models above but contained only fixed effects.
500 The resulting VIF measures collinearity in fixed effects. Craney and Surles (2002) suggest that
501 appropriate cutoffs for VIF range from 5-10. All ecological variables had VIF indices below 2.0
502 and were kept in all models (Supplemental Table 4).
503
504 We computed incidence rate ratios using the outputs of our GLMMs to examine the effects of
505 each predictor variable. For categorical variables, the incidence rate ratios represent the ratio of
506 the number of scans recorded in one level compared to the number of scans recorded in another
507 level. For variables with multiple levels (e.g. reproductive stage, dominance), a reference level is
508 selected and other levels are compared to the reference level to contextualise the effects of each
509 level on the response variable—in our case, resting scans or foraging scans. We plotted the
510 predicted outcomes for each reproductive stage using the plot_model function in the R package
511 sjPlot (Lüdecke, 2021). Visualising our data this way allowed us to identify patterns of resting
512 behavior or foraging behavior associated with reproductive stage while holding constant the
513 effect of all other predictors in our models.
514
515 *Fecal sampling for gut microbiota analysis*
516
517 We collected fresh fecal samples from study individuals 1-2 times per month within each
518 sampling period in 2014-2016. Once an animal defecated, we immediately collected the feces
519 into a sterile 2mL cryovial using personal protective equipment to minimize human

520 contamination of the samples. Fecal samples were visually inspected for dietary components,
521 many of which are identifiable by seed shape or arthropod exoskeletons remnants in the feces.
522 Dietary components were recorded to the most specific taxonomic classification possible.
523 Samples were stored on ice in insulated field packs for a maximum of 5 hours before being
524 transferred to a liquid nitrogen shipper (-90 C) for the remainder of the field season. If a sample
525 was suspected to be contaminated or compromised (e.g. falling on an area where a previous
526 defecation occurred), it was not collected. At the conclusion of each sampling season, samples
527 were shipped to the University of Calgary for processing. Samples were collected with
528 permission from the government of Costa Rica from CONAGEBIO (Approval No. R-025-2014-
529 OT-CONEGABIO) and exported under the Área de Conservación Guanacaste permit (DSVS-
530 029-2014-ACG-PI-060-2014). Samples were imported into Canada with permission from the
531 Canadian Food Inspection Agency (Import Permit: A-2016-03992-4). All data collection
532 complied with Costa Rican law and were approved by University of Calgary Animal Care
533 Committee (#AC15-0161).
534
535 *Laboratory processing*
536
537 Prior to DNA extractions, all laboratory equipment was bleached, autoclaved, and exposed to 60
538 minutes under UV light to minimize laboratory contamination. We randomized the order in
539 which samples were extracted to decrease bias associated with batch effects. Prior to DNA
540 extraction, we split each sample into two tubes, Extraction A and Extraction B to decrease
541 potential bias based on stochastic distribution of microbes within a fecal. During each round of
542 extractions, we extracted a laboratory blank to control for contamination from laboratory
543 surfaces or reagent. The extraction protocol included a bead-beading step to increase DNA yield
544 and specific optimizations are described in Orkin et al. (2019). We purified extracted DNA using
545 an Invitrogen PureLink PCR Purification kit (ThermoFisher Scientific Part No. K310001), after
546 which we combined extractions A and B prior to library preparation. Illumina amplicon
547 sequencing libraries were prepared in for the V4 region of the 16S rRNA gene at the University
548 of Minnesota following Gohl et al. (2016). Libraries were sequenced twice at the University of
549 Calgary to increase reads per sample on an Illumina MiSeq using v2 chemistry.
550

551 *Amplicon data preparation*

552

553 Raw reads were demultiplexed and sequencing barcodes and indices were removed using
554 cutadapt (Martin, 2011). We removed ambiguous base calls using the filterAndTrim function in
555 the R package DADA2, removed locus-specific primers using cutadapt, then determined quality
556 profiles using the plotQualityProfile (Callahan et al., 2016). Poor quality bases were truncated
557 again using the filterAndTrim function. Error rates were learned and dereplication was done
558 using learnErrors and derepFASTQ functions respectively. We merged forward and reverse
559 reads to generate amplicon sequence variants (ASVs). Chimeras were removed using the
560 removeBimeraDenovo function in DADA2, and we assigned taxonomies to ASVs using the
561 silva_nr_v132_train_set.fa file. We extracted and sequenced a series of negative lab controls,
562 which were then used to detect potential contaminants in the program decontam (Davis et al.,
563 2018). Using the function isContaminant, we compared the prevalence of sequences from the
564 negative controls with sequences from our samples. Using a 50% probability threshold, we
565 identified probable contaminants and removed them from our fecal sample sequences. We then
566 removed uncharacterized phyla, chloroplasts, and mitochondrial sequences from the dataset.

567

568 *Gut microbiota community structure*

569

570 To explore shifts in gut microbial community structure throughout the reproductive cycle, we
571 computed Chao species richness and Shannon alpha diversity for each sample. We removed 4
572 samples with Chao1 richness values >400 that were distinctly different that the remaining 304
573 samples, with Chao1 values ranging from 12-385. Because we sampled individuals multiple
574 times, and because sampling effort across individuals was uneven, we fit linear mixed effects
575 models to examine the relationship between reproductive state and richness and diversity
576 metrics. We included individual identity as a random effect in both models and included rainfall
577 and maximum temperature as ecological predictors. We used an alpha of 0.05 as a significance
578 cut-off.

579

580 We then removed extremely low-prevalence phyla for the remainder of analysis and filtered out
581 taxa that were not present in at least 5% of samples. Due to sample size constraints, we were not

582 able to divide fecal samples into subsets within reproductive states and therefore proceeded with
583 the categories cycling, pregnant, and nursing. To explore the relationship between reproductive
584 state and gut microbial community dissimilarity within our sample set, we transformed sample
585 counts to relative abundances and then computed Bray-Curtis dissimilarity values using the
586 ordinate function in phyloseq. We visualized beta diversity using non-metric multidimentional
587 scaling (NMDS). We used the function adonis in the R package vegan to run a PERMANOVA
588 to examine predictors of Bray-Curtis dissimilarities in our dataset (Dixon, 2003). In this
589 PERMANOVA, we included reproductive status as our predictor of interest, as well as
590 individual identity, rainfall, and dietary category based on fecal contents, as we suspected these
591 could be related to microbial community dissimilarity.
592
593 *Differential abundance*
594
595 To examine which, if any, bacterial taxa were differentially abundant among reproductive states,
596 we agglomerated samples at the genus level, then used the R package DESeq2 to compute
597 variance stabilized counts (Love et al., 2014). We then used Wald tests to determine the log2
598 fold differences among the reproductive states and used adjusted *P* values (alpha = 0.01) to
599 account for multiple tests. We conducted pairwise comparisons between cycling and pregnant
600 females, pregnant and nursing females, and nursing and cycling females to examine how these
601 transitions might be related to gut microbial community structure. We repeated this analysis for
602 all bacterial phyla in our dataset to examine courser scale shifts in fecal microbial community
603 structure among reproductive states.
604
605 *Estimated metabolic pathways*
606
607 We used the package PICRUSt2 to estimate metabolic pathways present in our samples using
608 KEGG orthologs (Douglas et al., 2020). We tested for significant dissimilarity in estimated
609 metabolic pathways among the reproductive states using a PERMANOVA including individual
610 identity as a control. We then used the linear discriminant analysis (LDA) effect size method
611 using the LEfSe package (Segata et al., 2011), which identifies the functional metabolic
612 pathways likely to explain differences between reproductive states and stages in our data. We

613 used a logarithmic LDA score of 2 as a cut off for discrimant features, and individual identity
614 was included as a predictor to account for individual variation. All code used for analysis in this
615 study is available at
616 https://github.com/webbshasta/CapuchinReproductionBehaviourMicrobiome.
617

618 **ACKNOWLEDGEMENTS**

619 We thank: Roger Blanco and the Área de Conservación Guanacaste for permitting this research;
620 Saul Cheves Hernandez, Monica Myers, Kelly Kries, Nuria Ferrero, Nile Carrethers, and Ronald
621 Lopez for assistance in the field; and Urs Kalbitzer and Jeremy Hogan for help with analyses.
622 The project that gave rise to these results received the support of a fellowship from "la Caixa"
623 Foundation (ID 100010434) and from the European Union's Horizon 2020 research and
624 innovation programme under the Marie Skłodowska-Curie grant agreement No 847648 (JDO).
625 The fellowship code is LCF/BQ/PI20/11760004. Research was also supported by The Eppley
626 Foundation for Research (JDO, ADM), The International Center for Advanced Renewable
627 Energy and Sustainability (I-CARES) (JDO, ADM), Washington University in St. Louis (JDO,
628 ADM), the Alberta Children's Hospital Research Institute (ACHRI) (ADM, SEW, JDO), the
629 National Sciences and Engineering Research Council (ADM), a Canada Research Chair Tier II
630 (ADM), Sigma Xi The Scientific Honor Research Society (SEW), the American Society of
631 Primatologists (SEW), Alberta Innovates Technology Futures (SEW), and a Vanier Canada
632 Graduate Scholarship (SEW).

633 **COMPETING INTERESTS**

634 The authors declare no competing interests.

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Supplemental Table 1. Ethogram of behaviors for white-faced capuchin monkeys at Sector Santa Rosa, Costa Rica

Type of Behavior	Specific Behavior	Code	Description
Foraging	Forage: Insect (Extractive)	EFI	Tearing branches, ripping bark
	Forage: Fruit (Extractive)	EFF	Pounding, scrubbing, or breaking open fruits
	Forage: Flower	FFL	Feeding on flowers
	Forage: Fruit	FFR	Feeding on fruit
	Forage: Insect	FIN	Feeding on insects
	Forage: Other	FOT	Bromeliad leaves, pith, vertebrates
	Forage: Visually	VFO	Actively looking for food, including gleaning insects while moving
	Forage: Out of sight	FOS	Monkey is foraging but mouth and/or forelimbs are not visible
Resting	Rest (Solitary)	RES	Lying alone, not moving
	Rest (Social)	SRE	Not moving, lying down
Travel	Travel	TRA	Travel; moving very rapidly, not pausing for foraging or socializing
Social Affiliation	Social (Active)	SAC	Monkeys are affiliative; allogrooming
Social Aggression	Social (Aggressive)	SAG	Chasing, biting conspecifics
Other	Vigilant	VIG	Scanning intently at a long range (not for food)
	Drink	DRI	Drink
	Excretion	EXC	Excretion of feces, urine, or vomit
	Self-Directed	SDI	Auto groom
	Play	PLA	Play: biting, chasing, hitting, bouncing, pushing, pulling, etc.
	Other	OTH	Inter-group encounter, mobbing predator, sexual behaviour

Supplemental Table 2. Generalized linear mixed models for resting and foraging behaviours. Model results and incidence rate ratios were computed for each prediction.

Prediction	Generalized Linear Mixed Model	Results							
Females in periods of high energy demand (i.e., pregnancy, nursing) will rest more than females in periods of lower energy demand (i.e., cycling).	TotalRestingScans ~ DominanceCat + RepStateStage + Rainfall_cm + TempMax + MonthlyFruitBiomass + offset(log(TotalScans)) + (1 | Animal), data = dfzGrouped, family = poisson(link = "log"))	**Predictor**	**Estimate**	**Std. Error**	**Z-Value**	**P-Value**	**Incidence Rate Ratios**	**Confidence Interval**	**P-Value**
		(Intercept)	-1.2988	0.08671	-14.98	<2e-16	0.27	0.23 – 0.32	<0.001
		DominanceCatmid	-0.04233	0.06849	-0.618	0.5365	0.96	0.84 – 1.10	0.537
		DominanceCathigh	**-0.13081**	**0.06053**	**-2.161**	**0.0307**	**0.88**	**0.78 – 0.99**	**0.031**
		RepStateStagePregnancyStage1	-0.13774	0.10057	-1.37	0.1708	0.87	0.72 – 1.06	0.171
		RepStateStagePregnancyStage2	-0.04896	0.10391	-0.471	0.6375	0.95	0.78 – 1.17	0.638
		RepStateStagePregnancyStage3	0.08571	0.09624	0.891	0.3732	1.09	0.90 – 1.32	0.373
		RepStateStageNursingStage1	0.11913	0.08252	1.444	0.1488	1.13	0.96 – 1.32	0.149
		RepStateStageNursingStage2	-0.09301	0.10209	-0.911	0.3623	0.91	0.75 – 1.11	0.362
		RepStateStageNursingStage3	0.0879	0.10163	0.865	0.3871	1.09	0.89 – 1.33	0.387
		RepStateStageCycling_PostWeaning	0.11204	0.10777	1.04	0.2985	1.12	0.91 – 1.38	0.299
		Rainfall_cm	-0.04054	0.02174	-1.865	0.0622	0.96	0.92 – 1.00	0.062
		TempMax	**0.22587**	**0.02182**	**10.352**	**<2e-16**	**1.25**	**1.20 – 1.31**	**<0.001**
		MonthlyFruitBiomass	-0.01562	0.02175	-0.718	0.4726	0.98	0.94 – 1.03	0.473
Females in periods of high energy demand (i.e., pregnancy, nursing) will forage more than females in periods of lower energy demand (i.e., cycling).	TotalForagingScans ~ DominanceCat + RepStateStage + Rainfall_cm + TempMax + MonthlyFruitBiomass + offset(log(TotalScans)) + (1 | Group/Animal), data = dfzGrouped, family = poisson(link = "log"))	**Predictor**	**Estimate**	**Std. Error**	**Z-Value**	**P-Value**	**Incidence Rate Ratios**	**Confidence Interval**	**P-Value**
		(Intercept)	-0.54966	0.060256	-9.122	<2e-16	0.58	0.51 – 0.65	**<0.001**
		DominanceCatmid	0.016859	0.042153	0.4	0.6892	1.02	0.94 – 1.10	0.689
		DominanceCathigh	-0.030515	0.031026	-0.984	0.3253	0.97	0.91 – 1.03	0.325
		RepStateStagePregnancyStage1	0.053213	0.061225	0.869	0.3848	1.05	0.94 – 1.19	0.385
		RepStateStagePregnancyStage2	-0.003497	0.068042	-0.051	0.959	1	0.87 – 1.14	0.959
		RepStateStagePregnancyStage3	-0.02176	0.062772	-0.347	0.7289	0.98	0.87 – 1.11	0.729
		RepStateStageNursingStage1	**-0.132814**	**0.053359**	**-2.489**	**0.0128**	**0.88**	**0.79 – 0.97**	**0.013**
		RepStateStageNursingStage2	-0.043159	0.061833	-0.698	0.4852	0.96	0.85 – 1.08	0.485
		RepStateStageNursingStage3	-0.123121	0.067554	-1.823	0.0684	0.88	0.77 – 1.01	0.068
		RepStateStageCycling_PostWeaning	-0.100933	0.072291	-1.396	0.1627	0.9	0.78 – 1.04	0.163
		Rainfall_cm	**0.026021**	**0.013125**	**1.983**	**0.0474**	**1.03**	**1.00 – 1.05**	**0.047**
		TempMax	**-0.139173**	**0.016169**	**-8.608**	**<2e-16**	**0.87**	**0.84 – 0.90**	**<0.001**
		MonthlyFruitBiomass	**0.038289**	**0.016033**	**2.388**	**0.0169**	**1.04**	**1.01 – 1.07**	**0.017**

Supplemental Table 4. Linear mixed model outputs for richness and alpha diversity among fecal samples across reproductive states and PERMANOVA for Bray-Curtis dissimilarity among fecal samples.

Study Component	Model Description	Model	Results							
Chao1 richness among reproductive states	Generalized linear mixed model with negative binomal distribution	chao1~ ReproductiveStatus + scale(Rainfall) + scale(TemperatureMax) + (1|INDIVIDUAL), data=metadataFilt)	**Predictor**	**Estimate**	**Std. Error**	**Z-Value**	**P-Value**	**Incidence Rate Ratios**	**Confidence Interval**	**P-Value**
			(Intercept)	4.76545	0.05274	90.356	<0.0001	117.38	105.86 – 130.17	<0.001
			Nursing	-0.04053	0.09002	-0.45	0.65256	0.96	0.80 – 1.15	0.653
			Pregnant	-0.13895	0.09505	-1.462	0.14377	0.87	0.72 – 1.05	0.144
			scale(Rainfall)	**-0.11816**	**0.03632**	**-3.253**	**0.00114**	**0.89**	**0.83 – 0.95**	**0.001**
			scale(TemperatureMax)	0.01115	0.03721	0.3	0.76439	1.01	0.94 – 1.09	0.764
Shannon alpha diversity among reproductive states	Linear mixed model with Gaussian distribution	alphadiv~ ReproductiveStatus + scale(TemperatureMax) + scale(Rainfall) + (1|INDIVIDUAL), data=metadataFilt)	**Predictor**	**Estimate**	**Std. Error**	**T-Value**	**P-Value**	**Incidence Rate Ratios**	**Confidence Interval**	**P-Value**
			(Intercept)	2.64774	0.0438	60.445	--	--	2.56 – 2.73	<0.001
			Nursing	0.09473	0.07983	1.187	--	--	-0.06 – 0.25	0.235
			Pregnant	0.08834	0.08754	1.009	--	--	-0.08 – 0.26	0.313
			scale(TemperatureMax)	**0.07061**	**0.03513**	**2.01**	--	--	**0.00 – 0.14**	**0.044**
			scale(Rainfall)	-0.05154	0.03443	-1.497	--	--	-0.12 – 0.02	0.134
Bray-Curtis dissimilarity among reproductive states	PERMANOVA using adonis function R package vegan	distance(psState_filt, method="bray") ~ ReproductiveStatus + INDIVIDUAL + scale(Rainfall)	**Predictor**	**Df**	**Sums of Squares**	**Mean Squares**	**F-Value**	**R^2**	**P-Value**	**--**
			Reproductive Status	2	0.614	0.30706	1.239	0.00789	0.186	
			Individual	**28**	**8.618**	**0.30779**	**1.242**	**0.11071**	**0.005**	--
			scale(Rainfall)	**1**	**0.711**	**0.71059**	**2.8674**	**0.00913**	**0.003**	--

Supplemental Table 4. Generalized linear models to test variance inflation factor for ecological variables.

	Generalized Linear Model		GVIF	Df	GVIF^(1/(2*Df))
Variance inflation factor test for ecological variables in Resting Model	glm(TotalRestingScans ~ DominanceCat + RepStateStage + Rainfall_cm + TempMax + MonthlyFruitBiomass + offset(log(TotalScans)), data = dfzGrouped, family = "poisson")	DominanceCat	1.080238	2	1.019483
		RepStateStage	1.41904	7	1.025314
		Rainfall_cm	1.197425	1	1.094269
		TempMax	1.280846	1	1.131745
		MonthlyFruitBiomass	1.198814	1	1.094904
	Generalized Linear Model		GVIF	Df	GVIF^(1/(2*Df))
Variance inflation factor test for ecological variables in Foraging Model	glm(TotalForagingScans ~ DominanceCat + RepStateStage + Rainfall_cm + TempMax + MonthlyFruitBiomass + offset(log(TotalScans)), data = dfzGrouped, family = "poisson")	DominanceCat	1.080238	2	1.019483
		RepStateStage	1.41904	7	1.025314
		Rainfall_cm	1.197425	1	1.094269
		TempMax	1.280846	1	1.131745
		MonthlyFruitBiomass	1.198814	1	1.094904