Activity budget and gut microbiota remain largely consistent across reproductive states in wild, female capuchin monkeys

Shasta E. Webb[1], Joseph D. Orkin[2], Rachel Williamson[1], Amanda D. Melin[1,3]

1 Department of Anthropology and Archaeology, University of Calgary, Calgary, Canada
2 Institut de Biologia Evolutiva, Universitat Pompeu Fabra-CSIC, Barcelona, Spain
3 Department of Medical Genetics, Cumming School of Medicine, University of Calgary, Calgary, Canada

ABSTRACT

Energy demands associated with pregnancy and lactation have been a significant force in mammalian evolution. To mitigate increased energy costs associated with reproduction, female mammals have evolved multiple behavioural and physiological responses. Some species alter activity budget to conserve energy during pregnancy and lactation, while others experience changes in metabolic rate and fat deposition. Previous studies suggest that restructuring of gut microbiota with shifting reproductive states may help females increase energy harvest from foods, especially during pregnancy, while others have found little change in response to pregnancy or lactation. Here, we combined a robust set of >10,000 individual behavioural scans with >300 fecal samples collected longitudinally across multiple years from 33 known white-faced capuchin monkey females to examine the relationships among behaviour, gut microbiota composition, and reproductive state. We used a 16S-based amplicon sequencing approach and the DADA2 pipeline to anlayse gut microbiota diversity and putative functions. We found that reproductive state explained some variation in activity budget, but that overall resting behaviour was relatively stable across the reproductive cycle. We found evidence for increases in biotin synthesis pathways among microbes in lactating females, and that the alpha diversity of gut microbial bacteria was higher in pregnant females. Otherwise, the gut microbiota community structure and estimated functional pathways were not significantly different between reproductive states. These results suggest that capuchins in our population may have limited ability to mitigate energy costs through behaviour and microbiota and/or that ecological factors,

including food availability and temperature may constrain behavioural flexibility and/or gut microbial plasticity. These data contribute to a broader understanding of plasticity and constrainst at multiple levels in response to the physiological shifts caused by 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).

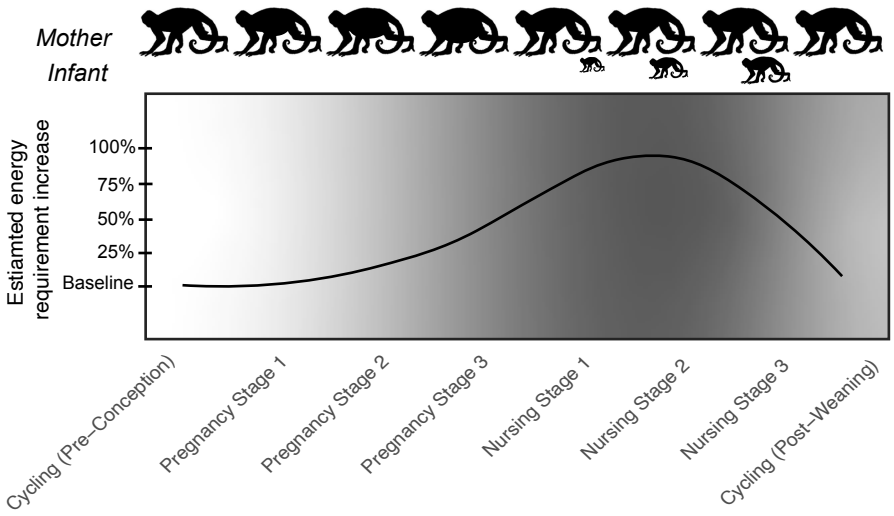

Figure 1. Visualization of estimated increases in energy requirements during the reproductive cycle of a non-human primate. Female primates face a 25% increase in daily energy requirements during gestation, and up to a 50-100% increase during lactation (Key & Ross, 1999).

Mammals vary widely in the length and subsequent energy costs of their reproductive cycles and have evolved multiple strategies in response. Adaptations include behavioural 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, behavioural 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 (*Cercopithecus 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)).

Adaptation to 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, 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 suggest 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 and shifts in prominent bacterial phyla associated with energy harvest, 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 during 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 the gut microbiome composition of females 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 respond to pregnancy is constrained or enabled by external factors.

Behavioural and microbial changes might interact to address the demands of pregnancy and lactation. However, few studies have combined behavioural 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 behavioural and gut microbial data from a well-studied population of wild non-human primates to examine the strategy or combinations of strategies that female primates employ to address the increased energetic costs

of pregnancy and lactation. To examine behavioural 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 3 years in a seasonal dry forest. We combine a robust data set of >13000 behavioural scans with >300 fecal samples collected from the same 33 monkeys to study behavioural and gut microbial responses to reproduction in a species that inhabits a dynamic 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 lactation, then females will rest more in these stages of higher energy demand. 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 predicted metabolic pathways related to energy metabolism and carbohydrate transport during pregnancy. Given the demonstrated potential for ecological and social factors to influence behavioural 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.

METHODS

Field site & study population

We collected samples and behavioural 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) accumulates (Melin et al 2020). Fruit abundance varies throughout the year and estimates of fruit biomass are calculated monthly

(Campos et al., 2015; J. D. Orkin et al., 2019). Sector Santa Rosa is home to three species of non-human primates: mantled howler monkeys (*Alouatta palliata),* black-handed spider monkeys (*Ateles geoffroyi*), and white-faced capuchins monkeys (*Cebus imitator*).

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). 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).

We collected data across 5 years (2014-2018). Non-invasive fecal samples were collected from 33 adult females from 4 social groups during multiple sampling bouts in 2014-2016. We collected behavioural data from 33 adult females from 4 social groups during multiple sampling bouts in 2016-2018. One female from the 2014-2016 fecal sampling periods disappeared from the population and was not included in behavioural analysis but was included in gut microbiome analysis. Two females reached sexual maturity during the 2016-2018 period and were included in behavioural analysis, but not in gut microbiome analysis. All animals in the study population are habituated to researcher presence and individually identifiable through physical markings on the face and body. In our dataset, 2016 was the only year in which we collected behavioural records and fecal samples simultaneously. Ideally, we would have behavioural records and fecal samples for all 5 years, but this was not possible due to field and laboratory limitations. However, the majority of individuals (31/33) were consistent across the entire study period and each dataset (behavioural and fecal) is robust and overlapping.

During bi-monthly censuses at the field site, reproductive state of each female is recorded. Pregnancies during the study period were determined via protrusion of the abdomen (visible approximately 8 weeks after conception) and we estimated conception dates when infants were born using 157 days as gestation length. We determined nursing on an *ad libitum* basis through observations of young monkeys suckling from adult females. Following Bergstrom (2015), we considered females nursing their own infants <12 months of age to be lactating. Juvenile capuchins are occasionally observed suckling after 12 months of age, but it is difficult to determine whether milk is transferred. We considered females that were never observed as nursing or pregnant on data collection days as non-pregnant, non-nursing. We grouped all non-pregnant, non-nursing females into the category "cycling" following Bergstrom (Bergstrom, 2015).

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 33 adult female capuchins is presented in Figure 2. Between 2014-2018, 43 infants were born in the study population. Behavioural data collection periods spanned portions of or the full duration of 40 of these pregnancies. Of the 40 infants that were born in the study period, 26 infants survived to weaning (~365 days), and behavioural data collection spanned portions of all 40 nursing periods, as well as captured transitions from nursing to non-nursing states. Between 2014-2016, 308 fecal samples from 29 individuals were collected. In 2016, fecal sampling overlapped with behavioural data collection.

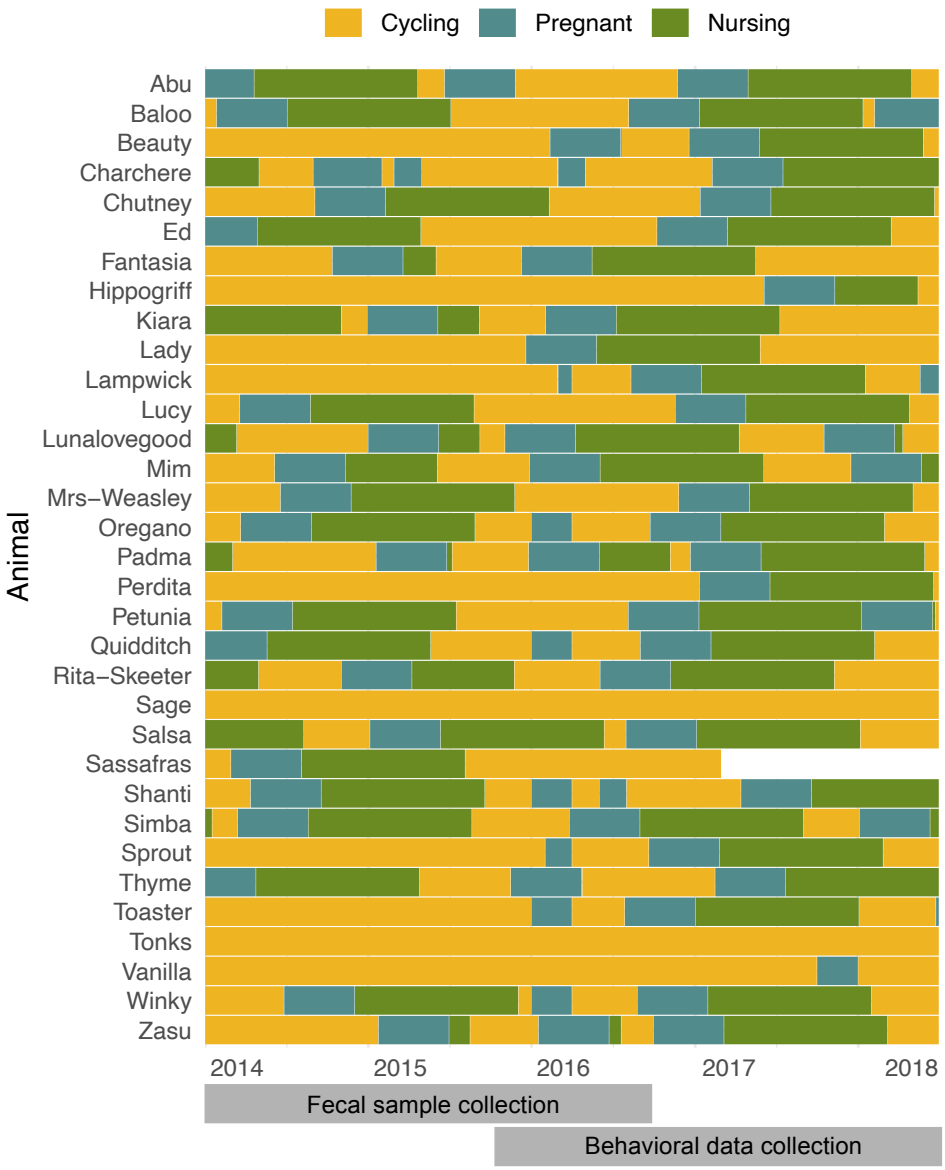

Figure 2. Reproductive status of study individuals observed between April 1, 2014 and June 30, 2018. Fecal samples were collected between April 29, 2014 and September 27, 2016. Behavioural data were collected between April 20, 2016 and June 22, 2018. One individual (Sassafras) disappeared from the population 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 blue (pregnancy) segments are followed by yellow (cycling) and not by green (nursing) segments. Green 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 behavioural state of each individual in the group (Supplementary 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.

Behavioural models for activity budget analysis

We fit a generalized linear mixed model (GLMM) that included reproductive stage as our predictor of interest. We also included monkey dominance rank, daily maximum temperature (°C) per day, 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) varied due to changing field conditions and stochastic movement and dispersal of group members. We included a log-transformed offset of total scans per animal per day to account for differences in sampling effort. Because our behavioural data are count data and because behavioural scans occur independently, a Poisson distribution with a logit link was designated in all models.

We tested whether our alternative model (fixed and random effects) outperformed the null model (random effects only). Likelihood ratio tests were conducted using the R function ANOVA. To test for multicollinearity between ecological variables a generalized linear model (GLM) was created to determine the variance inflation factor (VIF) (Craney & Surles, 2002). This model was identical to the alternative models above but contained only fixed effects. The resulting VIF measures collinearity in fixed effects. Craney and Surles (2002) suggest that appropriate cutoffs for VIF range from 5-10. All ecological variables had VIF indices below 2.0 and were kept in all models (Supplemental Table 2).

We computed incidence rate ratios using the outputs of our GLMM to examine the effects of each predictor variable. For categorical variables, the incidence rate ratios represent the ratio of the number of scans recorded in one level compared to the number of scans recorded in another level. For variables with multiple levels (e.g., reproductive stage, dominance), a reference level is selected and other levels are compared to the reference level to contextualise the effects of each level on the response variable—in our case, resting scans. We plotted the predicted outcomes for each reproductive stage using the plot_model function in the R package sjPlot (Lüdecke, 2021). Visualising our data this way allowed up to identify patterns of resting behaviour associated with reproductive stage holding constant the effect of all other predictors in our models.

Fecal sampling for gut microbiota analysis

We collected fresh fecal samples from known individuals 1-2 times per month within each sampling period in 2014-2016. Once an animal defecated, we immediately collected the feces into a sterile 2mL cryovial using personal protective equipment to minimize human contamination of the samples. Fecal samples were visually inspected for dietary components, many of which are identifiable by seed shape or insect shell remnants in the feces. Dietary components were recorded to the most specific taxonomic classification possible. Samples were stored on ice in insulated field packs for a maximum of 5 hours before being transferred to a liquid nitrogen shipper (-90 C) for the remainder of the field season. If a sample was suspected to be contaminated or compromised (e.g., falling on an area where a previous defecation occurred),

it was not collected. At the conclusion of each sampling season, samples were shipped to the University of Calgary for processing. Samples were collected with permission from the government of Costa Rica from CONAGEBIO (Approval No. R-025-2014-OT-CONEGABIO) and exported under the Área de Conservación Guanacaste permit (DSVS-029-2014-ACG-PI-060-2014). Samples were imported into Canada with permission from the Canadian Food Inspection Agency (import permit: A-2016-03992-4). All data collection complied with Costa Rican law and were approved by University of Calgary Animal Care Committee (#AC15-0161).

Laboratory processing

Prior to DNA extractions, all laboratory equipment was bleached, autoclaved, and exposed to 60 minutes under UV light to minimize laboratory contamination. The order in which samples were extracted was randomized to decrease bias associated with batch effects. Prior to DNA extraction, each sample was split into two tubes, Extraction A and Extraction B. Dividing samples in this manner decreases the chance of the "jackpot" effect, which may bias results based on stochastic distribution of microbes within a fecal sample. During each round of extractions, a laboratory blank was also extracted, to control for contamination from laboratory surfaces. The extraction protocol used included a bead-beading step to increase DNA yield and specific optimizations are described in Orkin et al. (2019). Extracted DNA was purified using an Invitrogen PureLink PCR Purification kit (ThermoFisher Scientific Part No. K310001), after which Extractions A and B were recombined prior to library preparation. Illumina amplicon sequencing libraries were prepared in for the V4 region of the 16S rRNA gene at the University of Minnesota following Gohl et al. (2016). Libraries were sequenced twice at the University of Calgary to increase reads per sample on an Illumina MiSeq using v2 chemistry.

Amplicon data preparation

Raw reads were demultiplexed and sequencing barcodes and indices were removed using cutadapt (Martin, 2011). We removed ambiguous base calls using the filterAndTrim function in the R package DADA2, removed locus-specific primers using cutadapt, then determined quality profiles using the plotQualityProfile (Callahan et al., 2016). Poor quality bases were truncated

again using the filterAndTrim function. Error rates were learned and dereplication was done using learnErrors and derepFASTQ functions respectively. We merged forward and reverse reads to generate amplicon sequence variants (ASVs). Chimeras were removed using the removeBimeraDenovo function in DADA2, and we assigned taxonomies to ASVs using the silva_nr_v132_train_set.fa file. We extracted and sequenced a series of negative lab controls, which were then used to detect potential contaminants in the program decontam (Davis et al., 2018). Using the function isContaminant, we compared the prevalence of sequences from the negative controls with sequences from our samples. Using a 50% probability threshold, we identified probable contaminants and removed them from our fecal sample sequences. We then removed uncharacterized phyla, chloroplasts, and mitochondrial sequences from the dataset.

Gut microbiota community structure

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

We then removed extremely low-prevalence phyla for the remainder of analysis and filtered out taxa that were not present in at least 5% of samples. Due to sample size constraints, we were not able to divide fecal samples into subsets within reproductive states and therefore proceeded with the categories cycling, pregnant, and lactating. To explore the relationship between reproductive state and gut microbial community dissimilarity and measure beta diversity within our sample set, we transformed sample counts to relative abundances and then computed Bray-Curtis dissimilarity values using the ordinate function in phyloseq. We visualized beta diversity using non-metric multidimentional scaling (NMDS).

Differential abundance

To understand specific components of gut microbial communities in our sample set, we selected the four most abundant phyla in the capuchin gut microbiome: Firmicutes, Actinobacteria, Baceteroidetes, and Proteobacteria. Previous research suggests these phyla change throughout reproduction (Koren et al., 2012; Mallott & Amato, 2018), and we wanted to examine if reproductive status predicted relative abundances of these phyla across our dataset. We used DESeq2 compute variance stabilized counts of ASVs within our phyla of interest (Love et al., 2014), then used Wald tests to determine the log2 fold differences (if any) between the reproductive states and adjusted *P* values (alpha = 0.01) to account for multiple tests. We repeated this analysis for all bacterial genera in our dataset.

Estimated metabolic pathways

We used the package PICRUSt2 to estimate metabolic pathways present in our samples using KEGG orthologs (Douglas et al., 2020). We tested for significant differences in estimated metabolic pathways between the reproductive states using a PERMANOVA. We then used the linear discriminant analysis (LDA) effect size method using the LEfSe package (Segata et al., 2011), which identifies the functional metabolic pathways likely to explain differences between reproductive states and stages in our data.

RESULTS

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

Resting activity within and among reproductive states

The model that included reproductive state outperformed a null model excluding this variable, suggesting some variation in resting behaviour was explained by reproductive stage. Predicted counts of resting scans per day are visualized in Figure 3 and demonstrate that resting increased

throughout pregnancy and early nursing, dipped in mid nursing, and increased again in late nursing. However, variation was mild and we did not find significant pairwise differences between the eight individual reproductive stages. High social rank was significantly negatively related to total resting scans (Estimate = -0.13, SE = 0.06, Z-Value = -2.16, p = 0.03). Maximum mean 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 3). Incident Rate Ratios for all predictors are presented in Figure 3 and values reported in Supplementary Table 3.

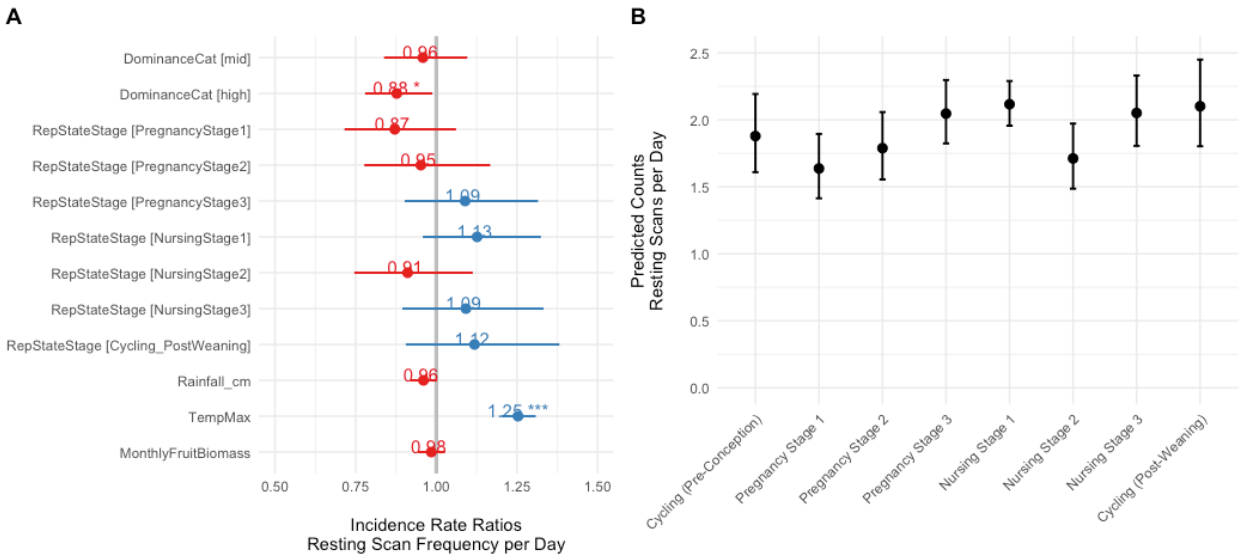

Figure 3. Incidence rate ratios (IRR) and standard error (**A**) for predictors from GLMMs of resting scans per day. The reference dominance category is low social rank; the reference reproductive stage is cycling (pre-conception). The grey vertical line represents "no effect". Values to the right of 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**).

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

Gut microbial community structure remained consistent among reproductive states

When we examined broad metrics of gut microbial community structure, we found that reproductive state was not a significant predictor of the species richness but it was a significant predictor of alpha diversity (Supplementary Table 4). Pregnant females exhibited a small but significant increase in alpha diversity (Estimate = 0.2, CI = 0.03 – 0.36, $p = 0.02$). Turning to gut microbial beta diversity, samples did not cluster distinctly according to reproductive state (Supplementary Figure 2).

Relative abundance of bacterial taxa remains consistent across the reproductive cycle

Pregnant females exhibited slight differences when we compared relative abundance of bacterial taxa. Specifically, among the four major phyla we examined (i.e., Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria), pregnant females exhibited a significant increase in the relative abundance of Firmicutes taxa compared to cycling females (Log2 Fold Change = 0.482, $p < 0.001$), although log2 fold change was less than 2. The relative abundances of Actinobacteria, Bacteroidetes, and Proteobacteria remained stable across the reproductive cycle (Figure 4). Variance stabilized values of bacterial genera abundance did not differ significantly among any of the reproductive states.

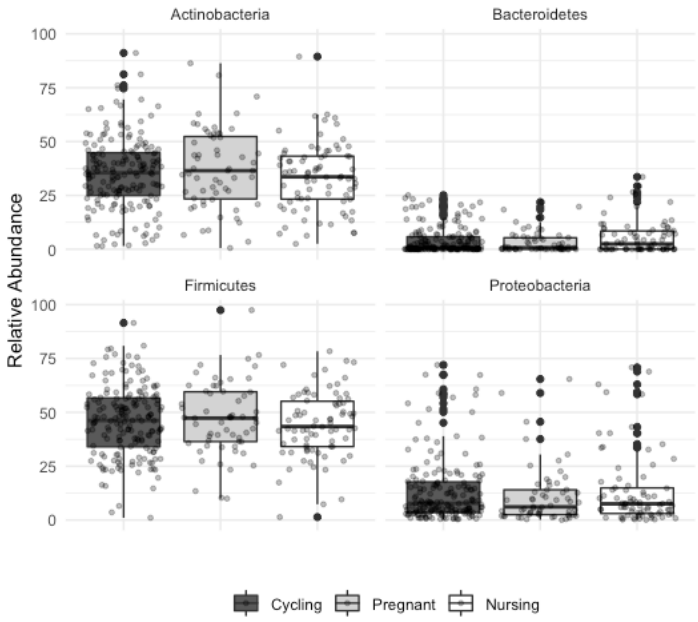

Figure 4. Bacterial taxa from the phyla Actinobacteria, Bacteroidetes, and Proteobacteria remained stable across the reproductive cycle. Taxa from the phylum Firmicutes were significantly more relatively abundant during pregnancy compared to cycling (Log2 Fold Change = 0.482, $p < 0.001$). Each point represents a single fecal sample.

Estimated metabolic pathways remain largely stable among reproductive states

We predicted that estimated metabolic pathways related to carbohydrate transport and energy uptake would increase during pregnancy. However, reproductive status was not a significant predictor of estimated metabolic pathways when all states were included (PERMANOVA; df = 2, F Value = 1.679, $p = 0.08$). In pairwise comparisons between reproductive states, the nursing state was a significant predictor of variation in estimated metabolic pathways (PERMANOVA; df =2, F Value = 2.748, $p = 0.028$). Specifically, nursing females were characterized by a significant increase in pathways related to biotin metabolism (Linear Discriminant Analysis effect size = 3.16, $p = 0.009$).

Discussion

Females maintain consistent resting behaviour throughout pregnancy and nursing

We analyzed a robust set of >13,000 individual scans to explore behavioural responses to reproduction and 308 fecal samples to understand gut microbial community dynamics in population of reproductively mature female capuchin monkeys. In our first aim we sought to compare activity budgets of white-faced capuchins among and within cycling, pregnancy, and nursing stages at a fine scale. Resting behaviour was largely consistent across the reproductive cycle, but the variation observed suggests that resting increases slightly during pregnancy and decreases in mid nursing. This pattern fits well with expectations from available data on energy expenditure (Figure 5). While no single tage in the reproductive cycle was stastically significantly different from the other stages, we see a general pattern that suggests resting increases during pregnancy and early lactation, before dipping in mid-lactation. The energy demands of this mid-lactation phase may require additional foraging behaviour, which reduces resting, a hypothesis that invites future testing. Overall, we did not find strong support for our prediction that females increase resting to mitigate energy costs of pregnancy and lactation. 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 behaviour may be too subtle to reach significance with present sample sizes. Capuchins may alter behaviour in other ways to cope with changing energy needs. For example, past research suggests that lactating females increase feeding rate (McCabe & Fedigan, 2007). There might also be underlying hormonal or other physiological changes that we were not able to capture in the present study that help pregnant and lactating females address energy costs.

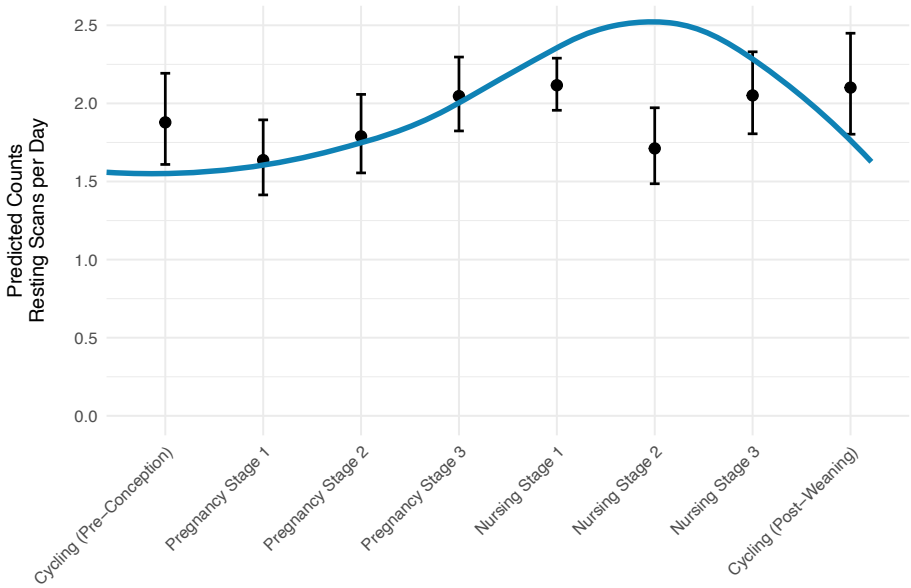

Figure 5. Estimated pattern of energy requirements (blue line) overlaid on predicted counts of resting scans.

While capuchins are generally considered highly flexible and plastic in response to changes in their environments (Fragaszy et al., 2004), it is possible the resting behaviour is constrained in this population, and 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 behaviours to overlap with available food and water (Campos & Fedigan, 2009). We also see changes in thermoregulatory behaviours; capuchins rest more during the hottest parts of the day in the hotter season of the year, and exhibit seasonal behaviours likely linked to thermoregulation and water consumption. We conclude from the results of our study and consideration of previous research that ecological, and to a lesser extent social, constraints preclude a large degree of behavioural flexibility during reproduction in this population.

Gut microbial communities remain largely stable throughout reproduction

We did not find strong support for the prediction that females modulate the gut microbiome to increase energy absorption from food during pregnancy and nursing. Females in cycling,

pregnant, and nursing states did not cluster separately in a beta diversity plot, suggesting alternative drivers of community dissimilarity. We observed a small but statistically significant increase in alpha diversity in pregnant females, which contrast previous studies in humans that showed a drastic decrease in alpha diversity (Koren et al., 2012). While, overall, female capuchins did not exhibit large gut microbial structural shifts, we did find that pregnant females exhibited small but significant increases in Firmicutes taxa. At a broad scale, taxa within Firmicutes are responsible for breaking down carbohydrates than endogenous 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).

In addition, lactating capuchins in our population exhibited increase in biotin metabolism pathways 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. Though specific dietary requirements for biotin are unknown for humans and other mammals throughout reproductive, a recent study of gestating and lactating women found that they require more biotin than current clinical recommendations (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 absorption.

Our results differ from previous studies of non-human primates, which suggest that the gut microbiome shifts considerably throughout the reproductive cycle (Amato et al., 2014; Mallott et al., 2020; Mallott & Amato, 2018). Mallott and Amato (2018) examined how gut microbial communities changed across reproductive states in 5 female capuchins 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 wet aseasonal forest, with little variation in food and water availability throughout the annual cycle (Mallott et al., 2018). The biome where our study took place is, by contrast, highly seasonal, with distinct shifts in temperature, water availability and fruit and arthropod abdunance (Campos et al., 2015; Mosdossy et al., 2015). In the hot, dry season animals contend with drought and high temperature, while in the rainier, cooler season, these forces are less present. We have observed strong effects of seasonality on ranging behaviour, 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). The effects of seasonality on this population of capuchins may be limiting behavioural and gut microbial responses to other factors, including reproduction.

Alternatively, we may be prematurely dismissing the importance of individual variation in response to reproductive states. For example, humans 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). High inter-individual gut microbiota among members of the same population has been found in humans (Healey et al., 2017; Zhu et al., 2015). Further, while activity budgets and amplicon sequencing provide important, thought relatively course, data about behaviour and gut microbiota respectively, future research on this population could incorporate individual focal data and/or shotgun metagenomic sequencing, both of which would provide a more detailed understanding of capuchin reproductive behavioural and microbial ecology.

Conclusions and future directions

The implications of these contrasting results within the same species are important when considering behavioural and gut microbial flexibility across a species range. Capuchins are among the most flexible and generalist species of platyrrhine primates (Fragaszy et al., 2004); however, the reality might be a more nuanced. Johnson and 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, chiefly temperature

range. Where our study took place is near the far edge of the capuchin range, both in terms of geography and temperature, which may explain some of the lack of flexibility that we see in behaviour 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.

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