Title: A COMPARISON OF FECAL GLUCOCORTICOID METABOLITE CONCENTRATION AND GUT MICROBIOTA DIVERSITY IN BONOBOS (*PAN PANISCUS*)

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Abstract

Host sex, age, diet, stress, and social environment have all been found to influence the gut microbiota. In non-human primates (NHP), recent evidence from gorillas found fecal glucocorticoid metabolite concentration (FGMC) had no significant role in structuring their gut microbiota, but there was a significant differential abundance between family Anaerolineaceae and gorilla FGMC. This pattern has yet to be examined in other NHP, like bonobos (*Pan paniscus)*. We compared FGMC to 16S rRNA amplicons for 201 bonobo fecal samples collected in the wild across five months to evaluate the impact of stress, measured with FGMC, on the gut microbiota. Simpson's index was the only alpha diversity index to have a significant linear relationship with FGMC [$R^2 = 0.9643$, $F(4, 210) = 28.56$, $p = 0.0023$]. FGMC level explained 1.63% of the variation in beta diversity for Jensen-Shannon Distance, 2.49% for Weighted UniFrac, and 3.53% for Unweighted UniFrac using PERMANOVAs. Differential abundance models showed seventeen taxa that were significantly correlated with FGMC. We found that genus *SHD-231* in the family Anaerolinaceae was significant in our differential abundance model results, similar to western lowland gorilla abundance model results. These results suggest bonobos exhibit different patterns than gorillas in alpha and beta diversity measures and that members of the family Anaerolinaceae may be differentially affected by host stress across great apes. Incorporating FGMC into gut microbiota research can provide a more robust understanding of how stress impacts the gut microbiota of primates and humans and has important ties to overall host health.

1.1 Introduction

The gastrointestinal tract and gut microbiota play essential roles in host nutrition and health across mammals [1]–[3]. Perturbations and dysbiosis to gut microbial communities have been linked to diseases like obesity, diabetes, irritable bowel disorders, and certain cancers in humans [4]–[8]. At the same time, physiological stress, hereafter referred to as stress, has been found to have a negative effect on the gastrointestinal tract and associated microbiota [9]. The definition of stress is a threat to homeostasis and may elicit an adaptive or allostatic load response that can be chronic or acute [10]. Chronic stress is stress that is experienced over a longer time frame (e.g., days to years), while acute stress often only lasts for several minutes to hours [11], [12]. Stress, as used in the human and NHP literature, is often refereeing to psychosocial stress; however, we will be using stress to refer to generalized chronic stress that includes psychosocial stress but may have other causes. Stress has been linked to dysbiosis in the gut microbiota [13]–[17] and has also been implicated in mediating the communication between a host and their commensal gut microbiota [18], [19]. There is thus an increasing interest in how host-produced stress hormones interact with the gut microbiota [19].

This increasing interest in stress and the gut microbiota has primarily been examined in laboratory models, with very few studies looking at wild living mammals [15], [20]. In germ-free rats, the lack of a gut microbial community appears to increase a rat's behavioral and endocrine stress response [21]. Other studies in mice have linked depression symptoms, anorexia, and cancer to increased stress levels and disruptions in the murine gut microbiota [22]–[24]. One of the few studies in wild- living mammals found that in eastern grey squirrels when physiological indicators of stress were measured in conjunction with gut microbial composition, physiological stress better-explained gut microbiota diversity and composition than did environmental factors

[25]. Therefore, there seems to be a general mammalian pattern that suggests higher stress is associated with a more disrupted gut microbiome, but this pattern has not been well-studied in wild-living mammals.

Wild-living non-human primates (NHP) offer good models to examine these patterns of stress and the gut microbiota. Amongst NHP, bonobos (*Pan paniscus*) and other great apes exhibit a broader diversity in their gut microbes than populations of humans living across different societies and therefore make a good model for testing patterns of stress and changes in gut microbial diversity and composition [26], [27]. However, the consistency of the pattern described above has only recently been studied in some wild-living NHP and has yet to be assessed across many species. Recent evidence from western lowland gorillas found no relationship between measures of alpha and beta diversity and proximate measures of stress, suggesting stress may not have as large of an effect on NHP gut microbiota as it does in other species [10]. The same study in western lowland gorillas also found a positive correlation between FGMC and relative abundance of three different gut microbial taxa, thus suggesting that stress may select for increases in certain types of bacteria within the gastrointestinal tract [10]. These results from western lowland gorillas suggest that NHP may not have disruptions to the gut microbiota associated with changes in stress in other mammalian species, yet whether all NHP follow the established gorilla or mammalian pattern remains to be determined. Additionally, the amounts and effects of stress hormones vary across different groups of mammals and NHP, and may be different in closely related taxa.

Stress hormones, specifically glucocorticoids, are considered the principal chemical compounds involved in the stress system of mammals, including NHP [18], [28]. Glucocorticoids are excreted through feces, and fecal glucocorticoid metabolic concentrations (FGMC) are often

quantified as proximate measures of stress in most mammals, including NHP [18], [29]–[31]. FGMC are known to be related to chronic stress rather than acute stress [32], [33], thus capturing the stress an individual experienced in the preceding forty-eight hours from when a fecal sample was collected [34]. The relationship between the gut microbiota and hormonal systems has far-reaching implications for host physiology which helps elucidate co-evolutionary forces [35]. As measured through behavioral indicators, maternal stress showed increased *Lactobacillus* microbes found in the captive macaque (*Macaca mulatta*) gut microbiota (Bailey & Coe, 1999). Additional investigations have attempted to understand the role of host reproductive hormones as a factor influencing the composition and diversity of primate gut microbiota in Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*) [37]. Nevertheless, how a host's gut microbiota responds to various stress-based fluctuations during short-term variation in stress remains to be examined in many NHP, like bonobos.

While there has been an increase in investigations into NHP gastrointestinal tract microbiota for some species, research questions regarding the bonobo gastrointestinal tract microbiota are relatively new. Comparisons across great ape microbiota found a correlation between phylogeny and gut microbiota composition but failed to look at population-specific patterns [38]. Therefore, we need to understand how different bonobos compare to other primates. The most recent investigations into the bonobo gut microbiota concluded that malaria parasite infection did not affect gut microbiota composition [39]. while most older studies have examined bonobo gut microbiota in terms of their phylogenetic relationship concluding that great ape gut microbiomes recapitulate their evolutionary relationships and have species-specific microbes [38], [40]. None of these studies investigated how stress affects the composition and diversity of the bonobo gut microbiota. Thus, how stress and the bonobo gut microbiota interact

is not well understood for bonobos, and we aim to use bonobos as a model to test patterns of NHP stress and gut microbiota.

Stress has been studied in wild-living bonobos as it relates to sociality and socio-sexual behavior [41]–[44]. While sex-based differences in stress hormones have been observed in bonobos, our understanding of how wild bonobo FGMC co-varies with other non-invasive measures of the gastrointestinal tract microbiota composition and diversity remains limited. Bonobos are female bonded and male philopatric, and this social structure may contribute to the sex-based patterns in FGMC [45], [46]. Sex differences in bonobo cortisol have been quantified in the context of inter-group encounters, with both females and males exhibiting higher cortisol levels during intergroup encounters while males had overall higher levels of cortisol [47]. Additionally, captive bonobos exhibited a similar pattern where the single male had overall higher cortisol levels than the five other females [48]. However, most studies have not correlated FGMC with gut microbiota composition and we aim to fill this gap.

We predict that bonobo gut microbiota will exhibit a pattern similar to what was found in western lowland gorillas because gorillas are a large-bodied great apes and are the only wild-living great ape species in which these patterns have been examined. We predict that alpha diversity, or within individual diversity, will not explain FGMC. We expect to find several taxa for beta diversity, specifically the three taxa, family Anaerolineaceae, genus *Clostridium* cluster XIVb and genus *Oscillibacter* found in western lowland gorillas' gut microbiota, whose abundance is significantly explained by FGMC.

2.1 Methods

2.1.1 Study Site and Sample Collection

The research site is the Iyema field camp, located just north of the Lomako river at (00°55) North, 21°06) East) in the Democratic Republic of Congo (DRC). The site is mainly covered by primary forest in *terra firma* soil with some swamps [42], [49]. We followed bonobos to their night nests for data collection as part of the Antwerp Zoo's ongoing habituation efforts from June 2017 – October 2017. Night nest locations were marked, and each nesting site was revisited the next morning. We identified each bonobo as it exited the nest and collected approximately five grams of fecal sample into 50 mL tubes with 10 ml of RNALater for each individual in the nesting party [52]. The samples were stored in a cool, dry place until shipped to the Ting Laboratory at the University of Oregon. They were stored in a minus 20°C freezer until extraction. The remainder of each fecal sample after collection in RNALater was brought back to camp, dried using a camp stove, and placed into bags with desiccant for fecal glucocorticoid analysis. Thus, for each fecal sample, we can obtain data on gut microbiota composition and diversity and fecal glucocorticoid metabolite concentration (FGMC). We collected 218 paired fecal samples that were first stored in tubes with RNALater for the gut microbial analyses with the remaining portion of the fecal sample, then dried for hormonal analyses.

2.1.2 Data Collection

ELISA assays - FGMC

To evaluate FGMC, we analyzed 218 dried fecal samples in the Global Health Biomarker Laboratory at the University of Oregon using ELISA assays to quantify cortisol as a measure of FGMC. We used the Arbor Assay's DetectX® Cortisol Enzyme Immunoassay Kit as it is designed to be used on dried fecal samples. The Cortisol Enzyme Immunoassay Kit measures the glucocorticoid cortisol, and standard curves have been generated for this assay kit (Arbor

Assay's DetectX®). We included known controls provided for Cincinnati Zoo bonobos (N=5) for each plate run. Fecal samples were ground to a powder using a mortar and pestle, weighed out to the protocol's recommended ≥ 0.2 g. of fecal material, avoiding any plant or partially digested food material. Samples were then diluted (1:4) in assay buffer supplied in the 96-well DetectX® Cortisol Enzyme Immunoassay Kit from Arbor Assays (catalog no. K003-H5W) and assayed according to the kit manufacturer's instructions. The kit manufacturer reported the detection limit for this assay as 45.4 pg/mL. To control for shifts in circadian rhythm for FGMC, we used those samples collected under night nests to ensure all bonobo samples were from approximately the same time point. All plates were read using a BioTek microplate reader and analyzed with Gen5 software version 2.0. For the FGMC controls, 100 µl aliquots of re-constituted fecal samples from 5 zoo control samples were pooled, diluted (1:4) in assay buffer, and divided into seven aliquots. We then spiked six of the aliquots with 100 µl of standards such that each aliquot of the sample received one of the six concentrations of standard (1000, 500, 250, 125, 62.5, 31.2 pg/ml). One aliquot was left neat. Both the spiked and neat aliquots were assayed according to kit instructions (Arbor Assay's DetectX®).

16S sequencing – gut microbiota composition

We used the 218 RNALater preserved fecal samples to extract, amplify, and sequence microbial DNA. Total genomic DNA was extracted from each fecal sample using the QIAamp PowerFecal DNA kit (QIAGEN) in the Ting Lab at the University of Oregon. Negative controls were included in extraction batches to test for contamination. DNA extracts were quantified using a Qubit dsDNA HS Assay Kit protocol using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Samples containing at least 1.0 ng/µl were prepared and sent for amplification and sequencing of the V4 hypervariable region of the bacterial 16S ribosomal RNA at the Genomics

and Cell Characterization Core Facility at the University of Oregon following previously published methods [53], [54]. Barcoded amplicons were sequenced on a 150 PE V3 run on the Illumina MiSeq platform (Illumina, San Diego, CA). The resulting sequences were demultiplexed and denoised using DADA2, operational taxonomic units (OTUs) were assigned using the Green genes database, and quality filtering and assembly was done using the QIIME2 pipeline for microbial analyses [55, p. 2]. The amplicon sequence variants (ASVs) table was created for samples rarified to an average sampling depth of 79,058 reads per sample. We removed samples below 315 and above 100,000 reads per sample which accounted for 8.7% of the original dataset. We retained 201 samples with 26,010,213 reads. Negative control samples were included for each extraction, PCR, and library preparation. Any ASVs that appeared in these negative controls were removed from the 201 samples using the R package 'decontam'[56].

2.1.4 Data analysis

We tested sex, age, whether a female had an infant, FGMC, and FGMC level as our predictor variables with bonobo gut microbiota composition and diversity as the response variable. FGMC level was considered high if the value from the ELISA assay was over 7115 ng/pl and low if the value was under 7115 ng/pl cortisol based on previously established values for the bonobos at this field site [42]. Statistics were run in R version 4.0.2 [57]. We calculated three measures of alpha diversity using the 'vegan' package [58]: Chao's [59], Shannon's [60], and Simpson's [61]. We ran two-way ANOVAs against the predictor variables, sex, age, whether a female had an infant, and FGMC level, and we ran linear regressions for FGMC against Shannon's index, Simpson's index, and Chao's index to study alpha diversity or within

individual diversity. To examine the relationship between FGMC and bonobo gut microbiota, we ran a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations using the 'adonis' function in the R package 'vegan' [58] PERMANOVAs use the calculated beta diversity for the Jensen-Shannon Distance, Weighted UniFrac, and Unweighted UniFrac dissimilarity matrices taking the model predictors sex, age, whether or not a female had an infant, and FGMC sequentially (Table 1). We used Jensen-Shannon's Distance because it is a positive distance measure, and it clusters not similar levels not only due to phylogenetic relatedness but primarily around similar levels of unassigned fractionation [62], [63]. Weighted UniFrac is good at detecting the difference between groups when all samples have shared groups in common, while Unweighted UniFrac is more sensitive to those rare or non-abundant taxa ina community. [64]. Additionally, these measures are becoming common in human microbiome studies to examine human beta diversity [65]–[67]. It is of note that PERMANOVAs factor in the order in which variables are entered into the model. We also ran abundance models using the ANCOM R package to test whether a member of the gut microbiota varies with high (21,540 – 7,115 ng/pl and low concentrations (1,073 – 7,115 ng/pl) of FGMC [42], [68].

Table 1. Predictor variables included in analysis and collection method.

Predictor variable	**Factor levels**	**Collection method**	**Lab analysis**
Sex	Male, female, unk	Behavioral observations corroborated with genetic sexing assay	Sexing assay

Age	Infant, juvenile, sub-adult, adult, unk	Behavioral observations	--
Infant (Whether or not a female had an infant)	Yes, no, unk	Behavioral observations	--
FGMC	Continuous value	Non-invasive fecal sample collection	ELISA assays to quantify cortisol
FGMC level	High = over 7115 ng/pl; low = under 7115ng/pl cortisol	Non-invasive fecal sample collection	ELISA assays to quantify cortisol

3.1 Results

3.1.1 Alpha diversity- only Simpson's diversity index was significantly linearly related to FGMC

Simple linear regression was used to test the relationship between FGMC and three alpha diversity indices, Chao's, Shannon's, and Simpson's diversity index. The model we fitted was linear model = alpha diversity index ~ FGMC. Simpson's diversity index was the only index to

have a significant linear relationship with FGMC [$R^2 = 0.9643$, $F(4, 210) = 28.56$, $p = 0.0023$]. Both Chao's and Shannon's indexes were not significantly related to FGMC.

The two-way ANOVA results were not significant for Shannon's, Chao's, and Simpson's diversity indices against FGMC level (Figure 1). The two-way ANOVA results were all not significant for Shannon's, Chao's, and Simpson's diversity indices against sex, age, and whether or not a female had an infant (Figure 2).

3.1.2 Beta diversity – FGMC level significantly explained beta diversity for all three beta diversity metrics, Jensen-Shannon's Distance, Weighted UniFrac, and Unweighted UniFrac dissimilarity matrix.

The PERMANOVA results for FGMC were non-significant for the Jensen-Shannon Distance model with FGMC (Supplementary Table 1). The PERMANOVA results for FGMC level showed that it explained 1.63% (Figure 3) of the variation in beta diversity for the Jensen-Shannon Distance (Table 2).

The PERMAOVA for Weighted UniFrac dissimilarity matrix found that sex explained 1.87% (Figure 4A) of the variation in the bonobo gut microbiome for the model with FGMC, while FGMC level explained 2.49% (Figure 4B) for the Weighted UniFrac (Table 3).

The PERMANOVA results for FGMC were non-significant for the Unweighted UniFrac dissimilarity matrix (Supplementary Table 2). The PERMANOVA results for FGMC level showed that it explained 3.53% (Figure 5) of the variation in beta diversity for the Unweighted UniFrac dissimilarity matrix (Table 4).

Table 2. PERMANOVA results for Jensen-Shannon Distance dissimilarity matrices for FGMC level.

	df	**Sums of Squares**	**Mean Squares**	***F-value***	R^2	**p-value**
FGMC level	2	0.803	0.40165	2.3391	0.01632	0.001 ***
Sex	2	0.370	0.18503	1.0776	0.00752	0.203
Age	4	0.734	0.18351	1.0687	0.01492	0.228
Infant	2	0.430	0.21515	1.2530	0.00874	0.057

Table 3. PERMANOVA results for Weighted UniFrac dissimilarity matrices for FGMC and FGMC level.

	df	**Sums of Squares**	**Mean Squares**	***F-value***	R^2	**p-value**
FGMC	200	7.1662	0.035831	0.80628	0.65259	0.922
Sex	2	0.2056	0.102800	2.31326	0.01872	0.025 *
Age	3	0.1500	0.050003	1.12519	0.01366	0.287
Infant	1	0.0374	0.037445	0.84261	0.00341	0.415
FGMC level	2	0.2737	0.136828	3.6070	0.02492	0.005 **
Sex	2	0.1558	0.077892	2.0534	0.01419	0.063
Age	4	0.1153	0.028819	0.7597	0.01050	0.667
Infant	2	0.0805	0.040260	1.0613	0.00733	0.335

Table 4. PERMANOVA results for the Unweighted UniFrac dissimilarity matrices for FGMC level.

	df	**Sums of Squares**	**Mean Squares**	***F-value***	R^2	**p-value**
FGMC level	2	1.476	0.73785	5.1684	0.03535	0.001 ***
Sex	2	0.387	0.19360	1.3561	0.00927	0.171
Age	4	0.558	0.13951	0.9773	0.01337	0.435
Infant	2	0.352	0.17625	1.2345	0.00844	0.245

3.1.3 Analysis of community variance (ANCOM) results – seventeen taxa were significantly related to FGMC level.

We found 17 taxa whose abundance was explained by FGMC level after Bonferroni correction (Table 3; Figure 5).

Table 3. Taxa from ANCOM results were significantly related to FGMC level after Bonferroni correction. For all the listed taxa, we rejected the null hypothesis that the FGMC level does not significantly explain the abundance of the listed taxa. The taxa are listed by: Kingdom, Phylum, Class, Order, Family, Genus.

Classification of Amplicon Sequence Variant (ASV)	**W**
Bacteria, Firmicutes, Clostridia, Clostridiales, Unknown, *Unknown*	272
Archaea, Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae, *Methanobrevibacter*	268
Bacteria, Firmicutes, Clostridia, Clostridiales, Unknown, *Unknown*	266
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Unknown, *Unknown*	266

Archaea, Euryarchaeota, Thermoplasmata, E2, [Methanomassiliicoccaceae], *vadinCA11*	260
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Unknown, *Unknown*	255
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, RF16, *Unknown*	253
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Prevotellaceae, *Prevotella*	249
Bacteria, Chloroflexi, Anaerolineae, Anaerolineales, Anaerolinaceae, *SHD-231*	247
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, [Paraprevotellaceae], *Unknown*	247
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, S24-7, *Unknown*	247
Bacteria, Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, *Unknown*	246
Bacteria, Firmicutes, Erysipelotrichi, Erysipelotrichales, Erysipelotrichaceae, *RFN20*	241
Bacteria, Verrucomicrobia, Verruco-5, WCHB1-41, RFP12, *Unknown*	241
Bacteria, Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, *Unknown*	239
Bacteria, Firmicutes, Clostridia, Clostridiales, [Mogibacteriaceae], *Unknown*	235
Bacteria, Firmicutes, Clostridia, Clostridiales, Lachnospiraceae, *Butyrivibrio*	229

4.1 Discussion

We aimed to test the relationship between FGMC and the gut microbiota for bonobos and compare the results to those reported for western lowland gorillas [10]. We predicted that bonobo gut microbiota would exhibit patterns similar to what was found in western lowland gorillas where alpha diversity (or within individual diversity) and beta diversity (or between individual diversity) were not significantly explained by FGMC and FGMC level. The abundance model results found three taxa that were significantly correlated with FGMC[10]. We used three alpha diversity measures, Chao's, Shannon's, and Simpson's. We used two measures of beta diversity dissimilarity metrics, Jensen-Shannon’s Distance which measures the distance between distributions [62], [69], and Weighted UniFrac, which is a distance metric that accounts

for the abundance of observed taxa [70]. We then used ANCOM to look at the differential abundance of specific taxa within the gut microbiota and how they related to FGMC level.

We found a statistically significant linear relationship between FGMC and Simpson's diversity index, and Chao's and Shannon's diversity indexes were not significant against FGMC. All of our ANOVA results for all three alpha diversity measures were not significant. Our results are similar to what Vlčková et al., (2018) found in western lowland gorillas, where FGMC did not significantly explain alpha diversity for Shannon's and Chaos' diversity indices but did for Simpson's index.

Our beta diversity results found FGMC level significantly explains between individual variation in the bonobo gut microbiota for all three of the beta diversity metrics tested (Jensen-Shannon's Distance, Weighted UniFrac, and Unweighted UniFrac). The amount of variation explained by FGMC level was between 1.5 – 3.5%, depending on the beta diversity metric. However, FGMC was not statistically significant in explaining beta diversity for bonobos. Our results are similar to what Vlčková et al., (2018) found for FGMC, in that FGMC did not significantly explain bonobo gut microbiota. However, our results differ because when FGMC level was binned, there was a significant effect.

We found seventeen taxa that were significantly related to FGMC level for bonobos. This result was different from the Vlčková et al. (2018) results that found only three microbial taxa that have a significant linear relationship with FGMC in western lowland gorillas (family Anaerolineaceae; genus *Clostridium* cluster XIVb; genus *Oscillibacter* [10]). We found members of the family Anaerolineaceae in bonobo samples. Interestingly, one taxon, genus SHD-231, was significant in our differential abundance model results similar to the western lowland gorilla results that found a significant linear relationship between gorilla FGMC and

family Anaerolineaceae. We did find one genus of *Clostridium* in the 201 bonobos that we sampled; however, this genus of *Clostridium* was not a part of cluster XIVb and was not significant in our abundance model results. We found no genus *Oscillibacter* in our bonobo samples, nor did we detect any of the higher family level Oscillospiraceae. Therefore, bonobos and western lowland gorillas appear to be similar in that members of the family Anaerolineaceae may be differentially affected during periods of high stress.

Other taxa that we found that were specific to the bonobo abundance results are two different unknown bacteria in the order Clostridiales that are related to bacteria that are known to break down plant polysaccharides [71], [72], two members of the order Bacteroidales, including family S24-7, typically found in the mammalian gut microbiota [73], [74], and order Burkholderiales, which is a gram-negative bacterial group that is comprised of either facultative anaerobes or aerobic bacteria [75], [76]. We also found Ruminococcaceae and Mogibacteriaceae which have been found in the human gut microbiota [77], [78]. Other notable genus level associations included *Methanobrevibacter* sp., a proinflammatory pathobiont, [79], genus *vadinCA11* sp. and an unknown member of family RF16 associated with the rumen gut microbiota [80]–[82], *Prevotella* sp., which is associated with plant-rich diets, chronic inflammatory conditions and non-Western diets [67], [83], [84], and a *Butyrivibrio* sp. a well know butyrate-producing bacteria [85], [86]. These different patterns between the bonobo and western lowland gorilla suggest that there may be species-specific and temporal-specific effects of FGMC on the abundance of specific taxa in primate gut microbiota. However, it does appear that the family Anaerolinaceae may be particularly affected by stress in great apes. Most of these association data come from other mammalian lineages, and their functional role in the bonobo gut microbial community remains unknown without metagenomic sequences.

The differences in our results for bonobos compared to the results from western lowland gorillas could be explained in several ways. One of the significant differences between our study and the gorilla study is the sample size, which may contribute to the different patterns seen in bonobos compared to gorillas. Vlčková et al. (2018) had forty-two samples while our study had 201, which may account for the different patterns we see in the alpha and beta diversity results in bonobos compared to western lowland gorillas. Also, while both Vlčková et al., (2018) and our study had similar approaches in studying FGMC and the gut microbiome, our methods may have differed in what fecal glucocorticoid was used. Vlčková et al. (2018) state they used FGMC methodology from Shutt et al. (2012), which describes the quantification of four different types of FGMCs, only one of which is cortisol. Additionally, we used dried fecal samples to run on our ELISA assays, while Vlčková et al., (2018) used 90% ethanol for their FGMC analysis, perhaps giving slightly different FGMC results. Given that the only FGMC that our study used was cortisol, and we used a different method for preserving our fecal samples, these methodological differences may explain the differences in our results for bonobos compared to the western lowland gorillas.

Compared to humans, in NHP, it seems like there are particular taxa that change in abundance rather than broad associations between stress and the gut microbiota. Thus, we should look more specifically at the family Anaerolinaceae in future studies looking at stress and the gut microbiota. Incorporating FGMC and gut microbiota data can provide a more robust understanding of how stress impacts the gut microbiota of primates. Future directions for this work include adding metagenomic sequencing and metabolomic data to our dataset to incorporate more functional results. Additionally, we aim to incorporate dietary and social variables in future analyses of the bonobo gut microbiota.

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Statement of Ethics

This research was completed with the approval of the University of Oregon's Institutional Animal Care and Use Committee (AUP-17-10). Research abided by the International Primatological Society's Code of Best Practices for Field Primatology and the American Society of Primatologist's Principles for the Ethical Treatment of Human Primates and followed the legal requirements laid out by the DRC government.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Statements

Contributor Role	Role Definition
AJH, MLW, CMB, FJW	Ideas; formulation or evolution of overarching research goals and aims.
AJH, MLW, CMB, FJW	Development or design of methodology; creation of models.
AJH	Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data.
AJH, CBM, MLW, KJB	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection.
JJS, NT	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools.
AJH	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse.
AJH	Creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation).

Contributor Role	Role Definition
AJH, KJB, MLW, CMB, FJW, JJS, NT	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre- or post-publication stages.
AJH	Preparation, creation and/or presentation of the published work, specifically visualization/data presentation.
FJW	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.
FJW, MLW, NT	Management and coordination responsibility for the research activity planning and execution.
AJH, FJW, MLW, CMB	Acquisition of the financial support for the project leading to this publication.

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Supplementary Tables

Supplementary Table 1. PERMANOVA results for Jensen-Shannon Distance for FGMC.

	df	**Sums of Squares**	**Mean Squares**	***F-value***	R^2	**p-value**
FGMC	200	33.299	0.16650	0.87801	0.67662	1.000
Sex	2	0.420	0.20978	1.10625	0.00853	0.177
Age	3	0.675	0.22510	1.18705	0.01372	0.127
Infant	1	0.219	0.21871	1.15337	0.00444	0.149

Supplementary Table 2. PERMANOVA results for Unweighted UniFrac dissimilarity matrices for FGMC.

	df	**Sums of Squares**	**Mean Squares**	***F-value***	R^2	**p-value**
FGMC	200	28.060	0.140299	0.85262	0.67213	0.986
Sex	2	0.417	0.208492	1.26703	0.00999	0.196
Age	3	0.509	0.169553	1.03040	0.01218	0.350
Infant	1	0.092	0.091615	0.55676	0.00219	0.794

Graphical Abstract:

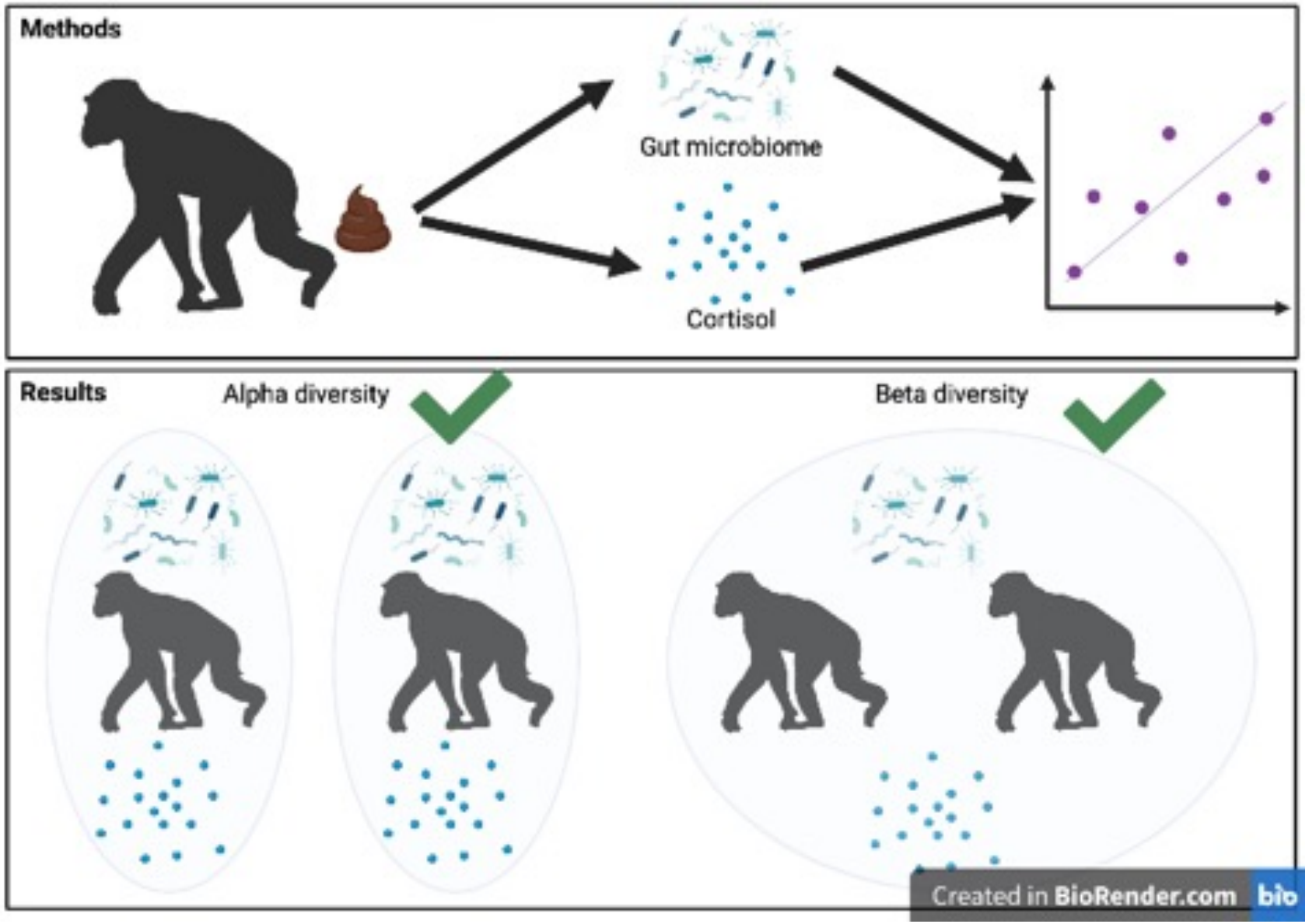

Figure 1:

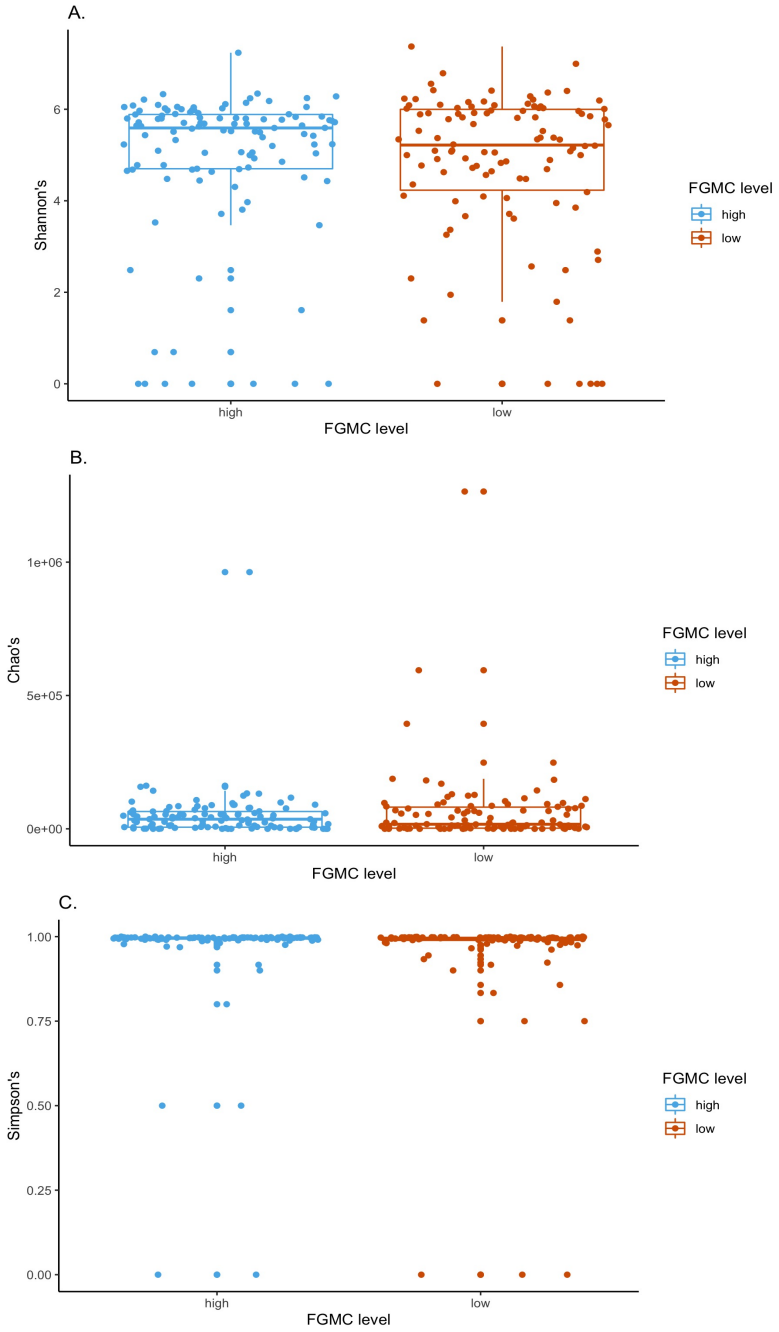

Figure 1. ANOVA results of FGMC level on A. Shannon's diversity index (F(2, 4, 2) = 5.312, p = 0.00542). B. Chao's, and C. Simpson's diversity index were not significant against FGCM level.

Figure 2:

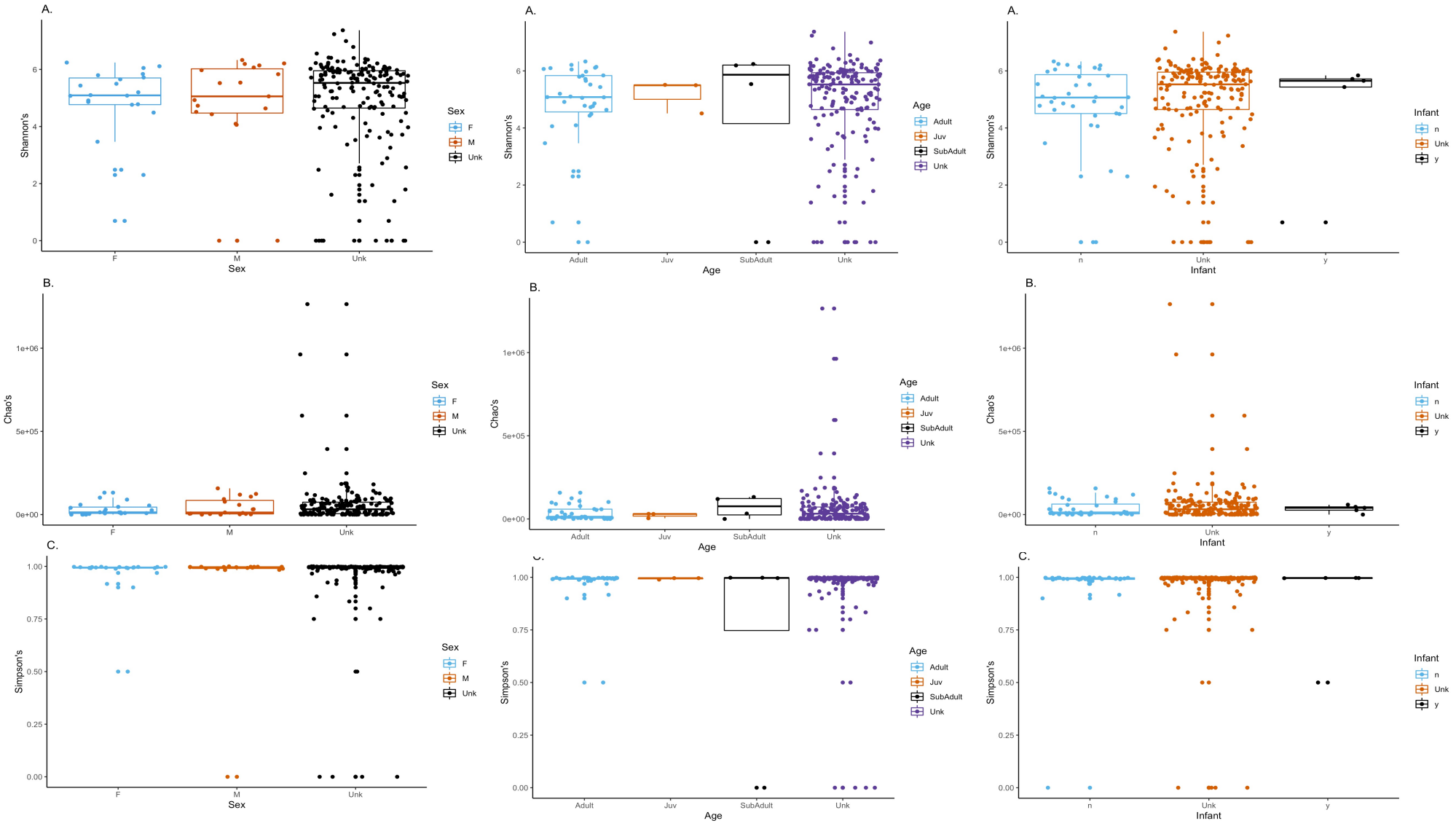

Figure 2. The predictor variables, sex (column 1), age (column 2), and infant (column 3) were all not significant for A. Shannon's, B. Chao's, and C. Simpson's diversity indices.

Figure 3:

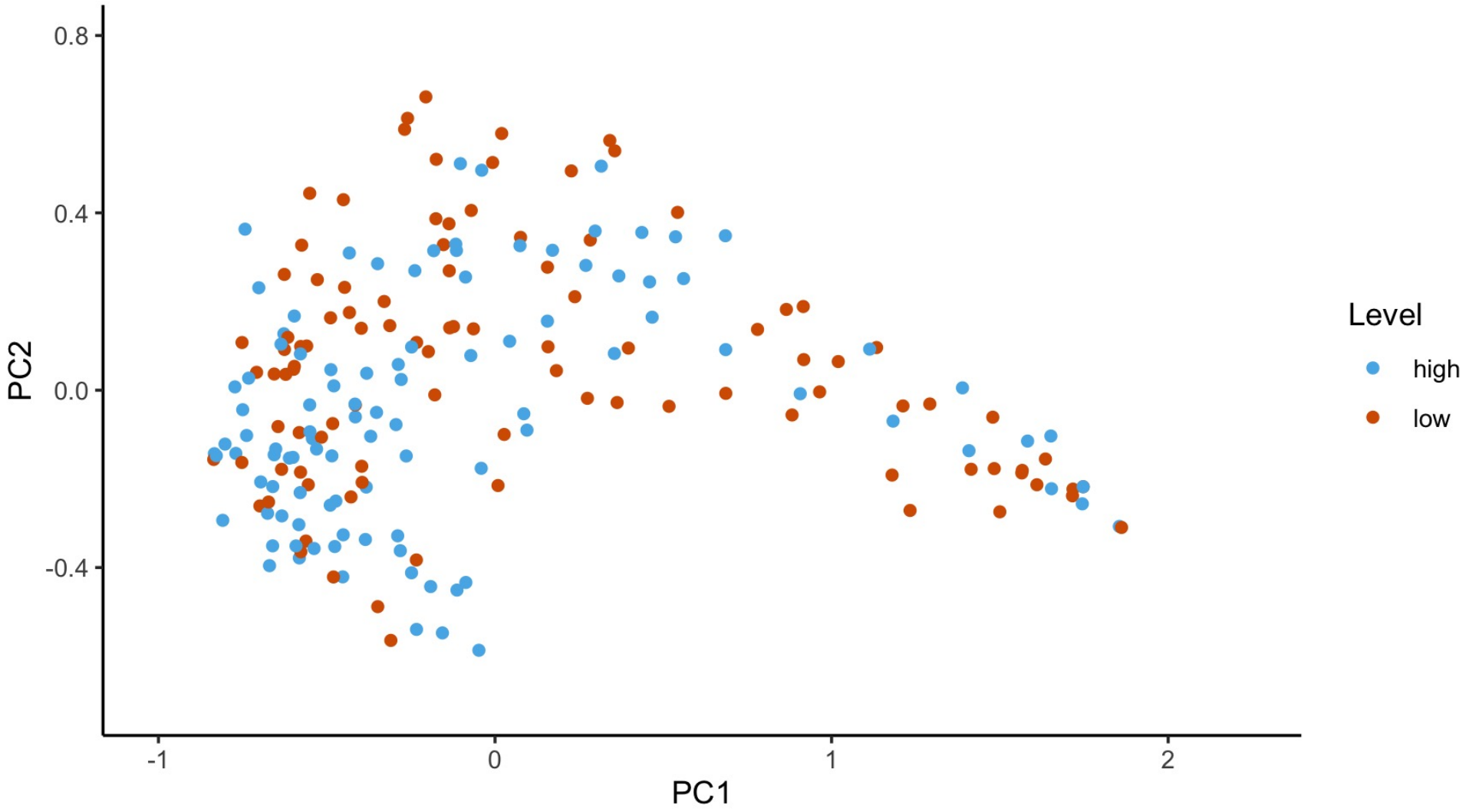

Figure 3. PCA plot of Jensen-Shannon Distance's matrix. The PERMANOVA results suggest that A. FGMC level explained 1.5% and B. whether a female had an infant explained 0.8% of the variation in beta diversity.

Figure 4:

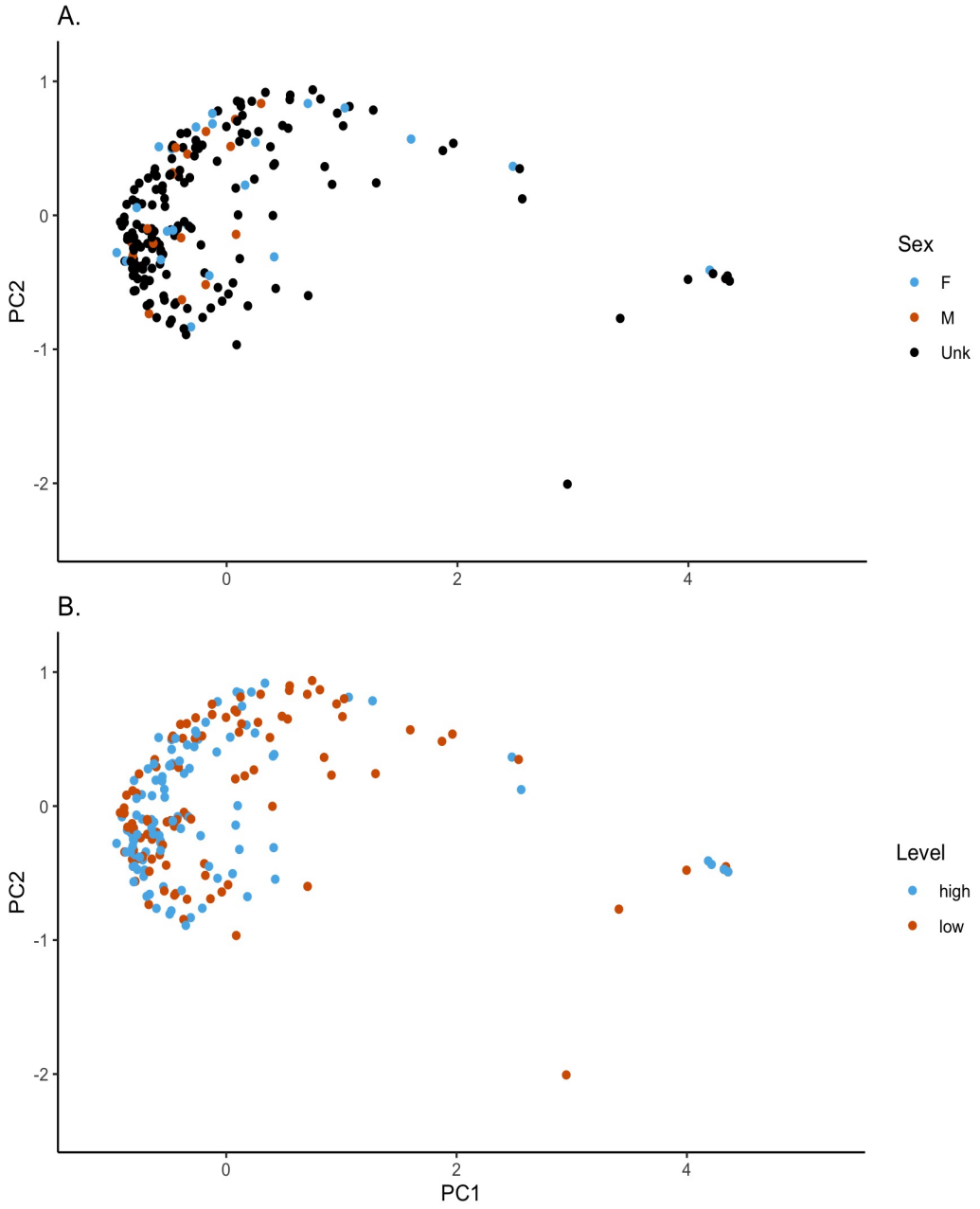

Figure 4. PCA plot of Weighted UniFrac's dissimilarity matrix. A. Sex explained 1.87% of the variation. B. FGMC level explained 2.49% of the variation in beta diversity.

Figure 5:

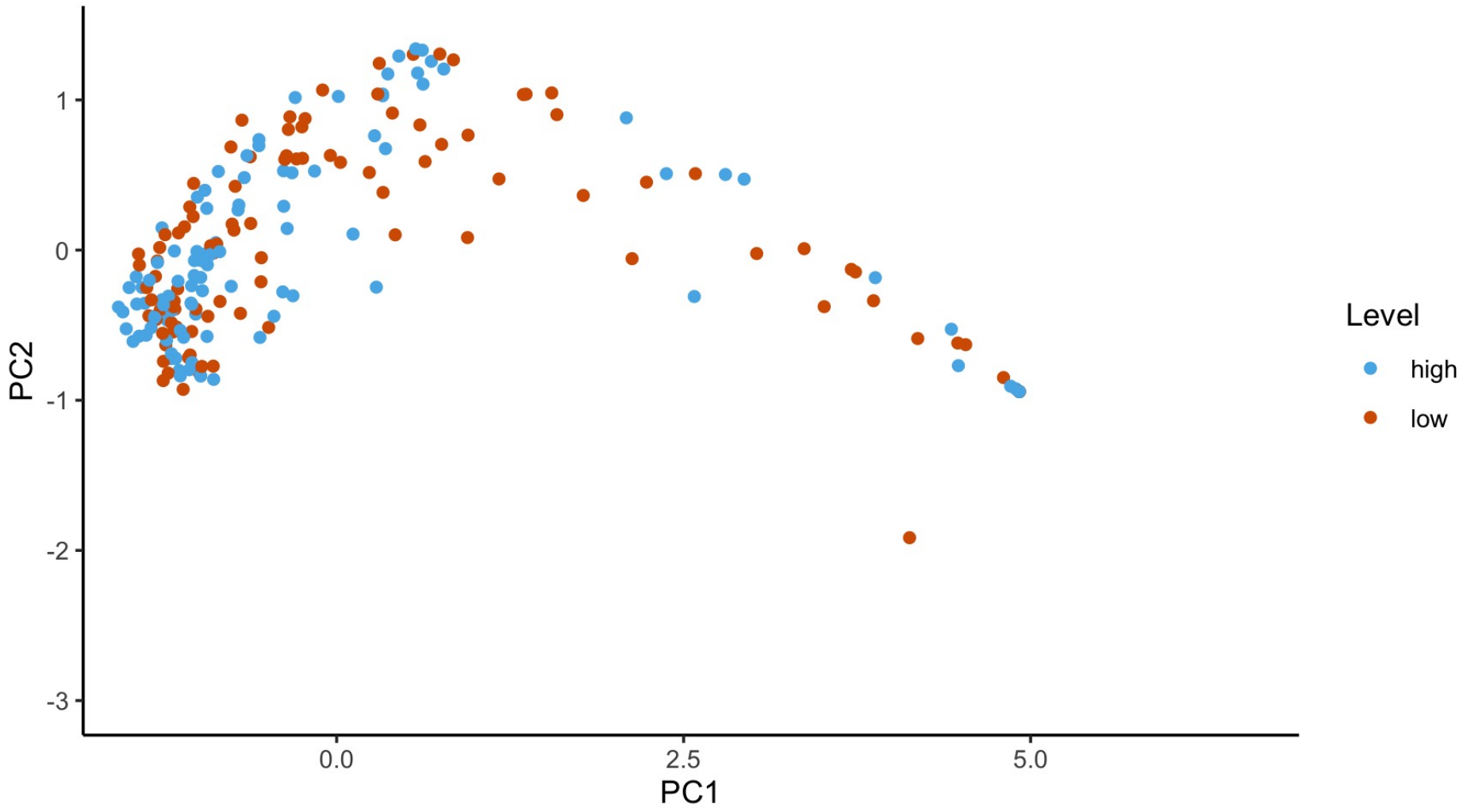

Figure 5. PCA plot of Unweighted UniFrac's dissimilarity matrix. FGMC level explained 3.53% of the variation in beta diversity.

Figure 5:

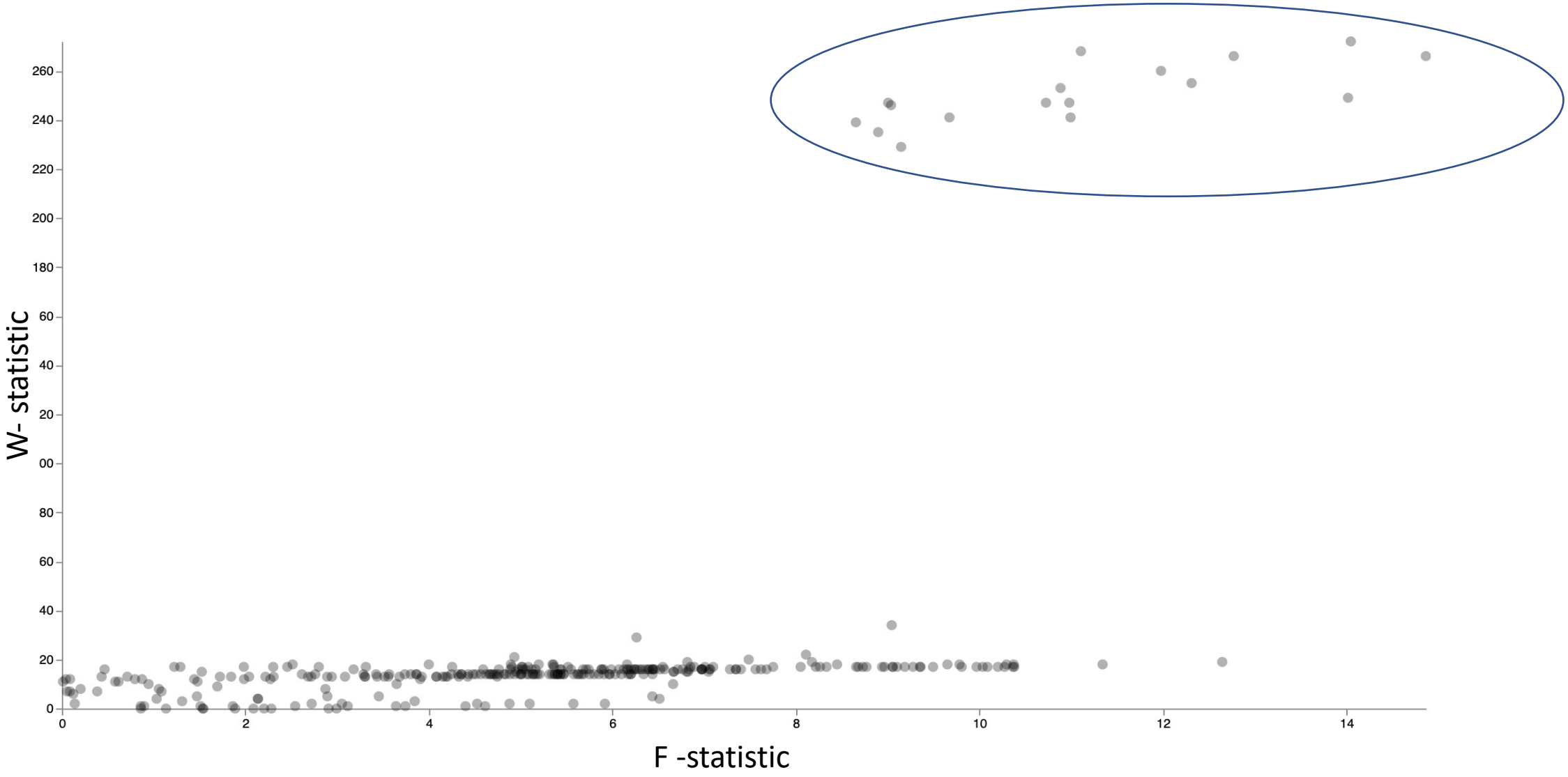

Figure 5. Volcano plot of W and F statistic for each ASV. 17 taxa (circled in blue) are significantly related to FGMC level.