Title

Gut Microbiota Uniqueness Is Associated with Lake Size, a Proxy for Diet Diversity, in Stickleback Fish.

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Abstract

Organismal divergence can be driven by differential resource use and adaptation to different trophic niches. Variation in diet is a major factor shaping the gut microbiota, which is crucial for many aspects of their hosts' biology. However, it remains largely unknown how host diet diversity affects the gut microbiota, and it could be hypothesized that trophic niche width is positively associated with gut microbiota diversity. To test this idea, we sequenced the 16S rRNA gene from intestinal tissue of 14 threespine stickleback populations from lakes of varying size on Vancouver Island, Canada, that have been shown to differ in trophic niche width. Using lake size as a proxy for trophic ecology, we found evidence for higher gut microbiota uniqueness among individuals from populations with broader trophic niches. While these results suggest that diet diversity might promote gut microbiota diversity, additional work investigating diet and gut microbiota variation of the same host organisms will be necessary. Yet, our results motivate the question of how host population diversity (e.g., ecological, morphological, genetic) might interact with the gut microbiota during the adaptation to ecological niches.

Introduction

Differential exploitation of resources, e.g., after colonization of a novel environment, is a driver of organismal divergence (Grant et al. 1976; Schluter 2000). Depending on resource availability and other ecological factors, species or populations can vary substantially in their ecological niche width; some are highly specialized (i.e., specialists) while others exploit a broader range of resources (i.e., generalists). However, generalist populations can be assemblages of specialized individuals (Araujo et al. 2011). Higher resource diversity can lead to broader ecological niche use through greater individual specialization and among-individual variation (Parent and Crespi 2009; Yurkowski et al. 2016). Differences in among-individual variation are associated with effects on population, community, and ecosystem dynamics (Schreiber et al. 2011; Start and Gilbert 2017; Vrede et al. 2011). An interesting next question is whether resource diversity as well as ecological diversity of animal populations is associated with the diversity of gut microbial communities (gut microbiota).

The gut microbiota strongly impacts host physiology, including nutrient metabolism (Sommer and Backhed 2013; Turnbaugh et al. 2006). In turn, a combination of host-associated (e.g., genetics, physiology, ecology) and environmental factors (e.g., temperature, salinity) shape gut microbial communities (Amato et al. 2019; Benson et al. 2010; Bresciano et al. 2015; Sepulveda and Moeller 2020; Spor et al. 2011). Of those, diet is particularly important, with short-term (e.g., seasonal) changes in diet (Bolnick et al. 2014c; Smits et al. 2017; Turnbaugh et al. 2009), and long-term adaptation to novel food sources (Baldo et al. 2017; Härer et al. 2020; Rennison et al. 2019; Youngblut et al. 2019), affecting the gut microbiota. Yet, little is known about how host-microbiota interactions facilitate ecological divergence and adaptation to novel trophic niches (e.g., Zepeda Mendoza et al. 2018). The magnitude of gut microbiota divergence can also be correlated with host phylogeny and genetic divergence (phylosymbiosis; Brooks et al. 2016). The confounding effect of host phylogeny can be largely avoided by studying closely related species or populations that independently adapted to similar niches. Such systems allow testing whether repeated

adaptive changes in trophic ecology across independent host lineages predict gut microbiota changes. Threespine stickleback fish (hereafter 'stickleback'), an important model system in evolutionary ecology, represent such a system with replicate adaptation and niche shifts seen among closely related lineages.

Stickleback are widespread throughout the Northern hemisphere (Bell and Foster 1994). Marine fish repeatedly colonized freshwater habitats, including lakes of varying sizes (Figure 1A), following glacial retreat 10,000 – 12,000 years ago. Lake stickleback adapted to mainly feed on two prey types: littoral invertebrates from the sediment (benthic prey) and pelagic zooplankton (limnetic prey) (Bell and Foster 1994), and sticklebacks' trophic ecology covaries with lake size on Vancouver Island, Canada. In association with resource availability, stickleback feed primarily on benthic invertebrates in small lakes whereas their diet is largely constituted of limnetic zooplankton in larger lakes (Bolnick and Ballare 2020). Interestingly, stickleback from intermediate-sized lakes occupy broader trophic niches on the population level, but not the individual level (Figure 1B&C). This repeated divergence in trophic ecology makes stickleback a powerful system to study gut microbiota dynamics in response to different diets. The stickleback gut microbiota has been increasingly studied over the last decade, and previous work has begun to characterize the contributions of host ecology and genetics to variation in gut microbial communities. Within-population gut microbiota variation is associated with diet, and effects depend on host sex and immune system (Bolnick et al. 2014a; Bolnick et al. 2014b; Bolnick et al. 2014c). Among populations, gut microbiota shifts have been detected in sympatric benthic-limnetic species pairs (Rennison et al. 2019), and gut microbiota differences are driven by host genetic divergence, habitat type, and geography (Smith et al. 2015; Steury et al. 2019). These findings suggest that assembly of sticklebacks' gut microbial communities is to some degree governed by host ecology and evolution.

We surveyed stickleback populations from 14 lakes to test whether gut microbiota diversity, divergence, and uniqueness is associated with lake size, a proxy for variation in trophic ecology (Bolnick and Ballare 2020). Bolnick and Ballare (2020) showed that trophic diversity is greatest in intermediate-

sized lakes but the extent of individual specialization does not vary with lake size. Thus, we hypothesized that (i) gut microbiota diversity is higher on the population level (gamma diversity), but not the individual level (alpha diversity) in intermediate-sized lakes compared to small and large lakes (Figure 1D), and (ii) among-individual gut microbiota uniqueness (alpha diversity/gamma diversity ratio) and divergence (beta diversity) are higher in populations from intermediate-sized lakes (Figure 1D). These results shed light on how host diet variation (inferred from lake size) might interact with the gut microbiota during adaptation to contrasting trophic niches.

Materials and Methods

Data collection

Samples were collected with minnow traps from lakes on Vancouver Island, Canada, in May and June of 2020 and 2021 under British Columbia Fish Collection permits NA20-602264 and MRVI21-619908, respectively. Lake sizes were obtained from Bolnick and Ballare (2020) and are based on data from the British Columbia Ministry of the Environment HabitatWizard and GoogleEarth satellite images for the smallest lakes. All fish were euthanized with an overdose of MS-222 (500 mg/L) and were stored at -20°C until dissection. Fish were rinsed with EtOH and whole guts were dissected using sterile equipment. Gut contents were removed by gentle squeezing to exclude transient bacteria and gut tissues were stored at -80°C until DNA extraction. DNA was extracted under sterile conditions in a laminar flow hood using the QIAGEN PowerSoil Pro Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). We amplified the V4 region of the 16S rRNA gene with barcoded 515F and 806R primers (https://github.com/SchlossLab/MiSeq_WetLab_SOP/blob/master/MiSeq_WetLab_SOP.md). PCR amplification was done in triplicate with a 10 µl reaction volume using the Platinum II Hot Start PCR Master Mix (Thermo Fisher Scientific), and the three replicates were subsequently pooled. Negative controls of sterile H_2O were included during DNA extraction and PCR, which did not yield detectable DNA concentrations. The PCR protocol consisted of an initial denaturation step for 60 s at 98 °C, 35 amplification

cycles with 10 s at 98 °C, 20 s at 56 °C and 60 s at 72 °C, and a final elongation at 72 °C for 10 min. Gel electrophoresis (2% agarose gel) was performed to visually check for amplification. DNA concentrations were measured on a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA), samples were pooled in an equimolar manner, and the completed libraries sequenced on the Illumina MiSeq 600 (PE300) platform at the UC Davis Genome Center after bead clean-up and quality check on a Bioanalzyer.

Gut microbiota analysis

We obtained a total of 11,906,277 raw sequencing reads (mean: 30,296 reads/sample; Table S1) that were imported into QIIME2 (Bolyen et al. 2019) for upstream analyses. Some samples had relatively low sequencing depths (Table S1) and filtering of reads during the merging of forward and reverse reads further decreased these numbers. To preserve coverage, we chose to use 250 bp of the forward reads, which had higher sequence quality, for downstream analyses. The QIIME2 plugin *dada2* was used to check sequence quality, correct sequencing reads, and filter chimeric sequences to obtain amplicon sequencing variants (ASVs) (Callahan et al. 2016). Across individuals, ASV richness ranged from 18-714 (median of 82), whereas median ASV richness ranged from 42.5-159 on the population level. A phylogenetic tree of bacterial lineages was constructed with FastTree 2.1.3 (Price et al. 2010). Bacterial taxonomy was assigned based on the SILVA 132 ribosomal RNA (rRNA) database at a 99% similarity threshold (Quast et al. 2013). We filtered out ASVs that (i) only occurred in one sample and had less than 10 reads, (ii) could not be assigned below the phylum level, or (iii) belonged to either chloroplasts, mitochondria, cyanobacteria, or archaea (Table S1). To avoid variation in gut microbiota diversity as an effect of sample size, we randomly sampled 24 host individuals per population (Table S1). After all filtering steps, we had an average of 18,445 reads per sample and 442,675 reads per population (Tables S1 & S2). We normalized our ASV table through scaling with ranked subsampling (SRS) with a C_{min} of 2599 reads (Beule and Karlovsky 2020).

We described gut microbiota diversity at the individual (alpha diversity) and population level (gamma diversity) as ASV richness, Shannon diversity, and Faith's phylogenetic diversity. We further calculated the

proportion of individual host diversity in relation to the total bacterial diversity of the same population (alpha diversity/gamma diversity ratio). We developed this measure to provide a novel estimate for the uniqueness of an individual's gut microbiota, where lower values indicate higher uniqueness and a smaller proportion of shared bacterial lineages among hosts of the same population. The alpha diversity/gamma diversity ratio is conceptually similar to beta diversity, which we calculated using three different metrics (Bray-Curtis dissimilarity, unweighted and weighted UniFrac) (Lozupone and Knight 2005; Lozupone et al. 2011). The UniFrac metrics consider phylogenetic relationships among bacterial lineages and weighted UniFrac further incorporates their relative abundances. We calculated each host's minimum beta diversity distance to its nearest neighbor within the same population, which represents another measure of gut microbiota uniqueness (Wilmanski et al. 2021). For each population, we calculated means of each diversity measure and performed quadratic regressions as a function of log lake size since a quadratic relationship between log lake size and within-population trophic diversity was previously established (Bolnick and Ballare 2020). We further tested whether geographic distance among lakes or genetic divergence among populations (based on FST values obtained from Bolnick and Ballare 2020) are correlated with differences in lake size or gut microbiota diversity using Mantel tests. All statistical analyses were done in R v4.2.1 (R Core Team 2021).

Results

Across 14 stickleback populations from lakes ranging in surface area from 4.4 to 2800 hectares (Figure 1A), we used quadratic regression to test for an effect of lake size (a proxy for variation in trophic ecology) on the gut microbiota. As predicted, there was no significant association between alpha diversity (mean within-population ASV richness of individual hosts) and lake size ($F = 0.30$, $r^2 = 0.05$, $P = 0.745$; Figure 2A). Contrary to our prediction, there was also no association between gamma diversity (cumulative population-level ASV richness) and lake size ($F = 0.12$, $r^2 = 0.02$, $P = 0.887$; Figure 2B). Results were qualitatively similar for Shannon diversity and Faith's phylogenetic diversity (Figure S1). Yet, using alpha

diversity/gamma diversity ratio as a measure of individual gut microbiota uniqueness, we detected a significant quadratic relationship with the lowest values in populations from intermediate-sized lakes based on ASV richness (F = 6.66, r^2 = 0.55, P = 0.013; Figure 2C), whereas the average proportion of ASVs that individuals share with their population ranged from 6%-7%. We further found suggestive evidence for a similar relationship for Shannon diversity and Faith's phylogenetic diversity (P < 0.1 for both metrics; Figure S1). These results indicate that gut microbiota uniqueness is highest in lakes where stickleback feed on more diverse diets, however, results were not confirmed when measuring uniqueness as minimum beta diversity distance (Figure S2). For mean beta diversity, we only found suggestive evidence for an effect of log lake size based on weighted UniFrac, which takes into account abundance and phylogenetic divergence of bacterial lineages (F = 2.97, r^2 = 0.35, P = 0.093; Figures 2D & S2). Among-population differences in any of the gut microbiota diversity measures or differences in lake size were not correlated with geographic distance among lakes or with genetic divergence among populations (Table S3), suggesting that our results were not confounded by these factors.

Discussion

Whether diet diversity at the population level promotes gut microbiota diversity (alpha and gamma diversity) as well as among-host uniqueness (alpha diversity/gamma diversity ratio) and divergence (beta diversity) remains largely unknown. Our study takes a first step by indirectly investigating this question in stickleback lake populations using lake size as a proxy for trophic ecology (also see Weinstein et al. 2021). These populations represent a compelling system to study gut microbiota diversity as stickleback show substantial and repeated adaptation to benthic and limnetic diets associated with lake size, as well as differences in within-population variation in their trophic ecology (Bolnick and Ballare 2020). Our results suggest that individual hosts show a higher gut microbiota uniqueness in intermediate-sized lake where stickleback occupy broader trophic niches, indicating that diet diversity might promote gut microbiota diversity. Yet, we inferred diet diversity from lake size based on a previous study that characterized

stickleback stomach contents (Bolnick and Ballare 2020), and their data was collected more than a decade before our sampling. We acknowledge that temporal changes in stickleback ecology and environmental conditions could affect our conclusions. Thus, we cannot make a direct connection between diet diversity and the gut microbiota, but our results provide important insight into the factors that likely shape the distribution of microbiota diversity and motivate follow-up studies.

It is now common knowledge that diet affects gut bacterial communities, as shown in a broad range of vertebrate hosts, including fishes (Baldo et al. 2017; David et al. 2014; Turnbaugh et al. 2009). A few studies found positive correlations between divergence in diet and gut microbiota composition (Härer et al. 2020; Li et al. 2016). Yet, the effects of diet diversity on gut microbiota diversity and divergence on the host population level remain largely unknown. Two studies have previously attempted to tackle this question in stickleback: Bolnick et al. (2014b) found that fish feeding on a mixture of benthic and limnetic prey items showed lower alpha diversity, both in the wild and in the lab. However, their study investigated only one population, leaving open the question of whether similar patterns would be expected across populations that vary in trophic niche width. Smith et al. (2015) addressed this and found some evidence for lower among-individual gut microbiota divergence (beta diversity) in intermediate-sized lakes. While these results contrast our findings of higher gut microbiota uniqueness in intermediate-sized lake, this discrepancy could be explained by differences in study design. Their study only investigated six lake populations, the largest lake was 330 hectares (our study included six lakes larger than that), only one beta diversity metric was tested, and the detected pattern was solely based on one data point (Smith et al. 2015). We aimed to overcome these limitations by studying associations between gut microbiota diversity and lake size more comprehensively across a larger dataset with more diverse lake sizes and by testing multiple diversity measures.

We found that gut microbiota diversity on the individual (alpha diversity) and population (gamma diversity) level were unaffected by lake size (Figure 2A&B). Notably, alpha diversity and gamma diversity

patterns were very similar, suggesting that mean alpha diversity is a good predictor for gamma diversity in our study system. The alpha diversity results were not unexpected since wider trophic niches in stickleback populations from intermediate-sized lakes were produced by greater among-individual variation rather than greater individual niche width (Bolnick and Ballare 2020). Further, associations between diet diversity and gut microbiota alpha diversity appear to vary strongly across host lineages (Bolnick et al. 2014b; Kable et al. 2022; Kartzinel et al. 2019; Weinstein et al. 2021). However, gamma diversity was also not associated with lake size (Figure 2B), which was contrary to our prediction. Variation in gut microbiota gamma diversity might be driven by other factors such as environmental heterogeneity, microbial diversity of the lake environment, or host genetic diversity, rather than by diet. While we did not specifically test for effects of these factors, a recent study on stickleback from the same lakes found evidence for higher genetic diversity in populations from larger lakes (Bolnick and Ballare 2020). Our results indicate that host genetic diversity is most likely not the main driver producing variation in gamma diversity across stickleback populations. Yet, gut microbiota composition is known to be partially controlled by host genetics (Goodrich et al. 2014; Macke et al. 2017), and especially by the genetic diversity of immune-related genes such as MHC (Bolnick et al. 2014a). Furthermore, genetic and geographic distance have been shown to be drivers of stickleback gut microbiota divergence (Smith et al. 2015; Steury et al. 2019). We did not detect evidence of significant correlations between genetic or geographic distance and differences in lake size or any of our gut microbiota diversity measures (Table S3), indicating that our conclusions are not confounded by geographic or genetic clustering of similarly sized lakes. Yet, the variation in alpha and gamma diversity remains unexplained and future studies should strive to identify the factors that shape population-level gut microbiota diversity.

To control for gamma diversity variation among populations, which might be produced by a range of different factors independent of stickleback's trophic ecology, we calculated the alpha diversity/gamma diversity ratio which we present as a novel measure of gut microbiota uniqueness. This is useful since

similar gamma diversity could be produced either by individuals having higher, but very similar alpha diversity, or by individuals having lower, but more unique, alpha diversity, and the alpha diversity/gamma diversity ratio captures such differences. In accordance with our prediction, we detected higher gut microbiota uniqueness (and suggestive evidence for higher divergence based on weighted Unifrac; Figure 2D) in individuals from intermediate-sized lakes (Figures 2C & S1). Yet, no such relationships were detected when using hosts' minimum beta diversity distances as another metric of uniqueness (Figure S2B). These inconsistent results might be due to these metrics capturing different aspects of gut microbiota diversity and composition. The alpha diversity/gamma diversity ratio provides information on the proportion of each host's alpha diversity in relation to the population's overall diversity. The minimum beta diversity metric merely captures how similar each host's gut microbiota is compared to the host with the most similar gut microbiota, therefore lacking information on the distribution of beta diversity values within a host population.

One interesting question is how the higher gut microbiota uniqueness (based on alpha diversity/gamma diversity ratio) in populations with higher diet diversity is produced and maintained by host populations. Diet has a major effect on the gut microbiota, and changes in diet have been shown to be associated with shifts in gut microbiota composition and diversity (Baniel et al. 2021; Bolnick et al. 2014b; Smits et al. 2017). Theoretically, the exploitation of more diverse resources, as encountered by stickleback in intermediate-sized lakes (Bolnick and Ballare 2020), could select for maintaining microbes important for nutrient metabolism and increased levels of gut microbiota specialization of individual hosts associated with their trophic specialization. Taken together, this could widen a host population's metabolic capabilities and might be adaptive in environments with high resource diversity. As a next step, it would be interesting to determine whether increased gut microbiota specialization in individuals from populations occupying broader trophic niches is accompanied by a higher capacity to rapidly shift gut microbiota composition in response to varying food sources. Such gut microbiota plasticity is thought to

allow hosts to exploit novel food sources, thereby potentially even affecting their evolutionary trajectories (Kolodny and Schulenburg 2020). Controlled lab experiments, including common garden studies, will be necessary to determine whether there are indeed population-specific differences in gut microbiota plasticity. While some studies have begun to determine the importance of gut microbiota plasticity for their hosts' ecology and evolution, characterization of among-species differences from a broader range of host lineages has the potential to greatly improve our comprehension of the capacity of different organisms to rapidly adapt and diversify.

Using lake size as a proxy for trophic ecology, our study is one of the first to suggest that diet divergence among individuals of the same population is reflected by increased gut microbiota uniqueness among hosts, but not by individual level (alpha) or population level (gamma) diversity or by gut microbiota divergence (beta diversity; except for suggestive evidence based on weighted UniFrac). While additional research incorporating gut microbiota and diet data of the same host individuals will be necessary before drawing firm conclusions, our results nonetheless suggest that diet diversity might promote gut microbiota diversity. This provides an important starting point for the further exploration of how diversity of resources, hosts (e.g., ecological, morphological, genetic), and their gut microbiota interact during the adaptation to different ecological niches. Studying the gut microbiota in an eco-evolutionary framework has the potential to improve our general understanding of the ecological and evolutionary consequences of host-microbiota interactions (Alberdi et al. 2016; Moeller and Sanders 2020).

Data and Code Availability

The raw sequencing data (https://figshare.com/s/09e18e90611a0de2316e), and data files and R scripts (https://figshare.com/s/18eed8d180f0e7417f14) are accessible from the figshare repository.

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Figures

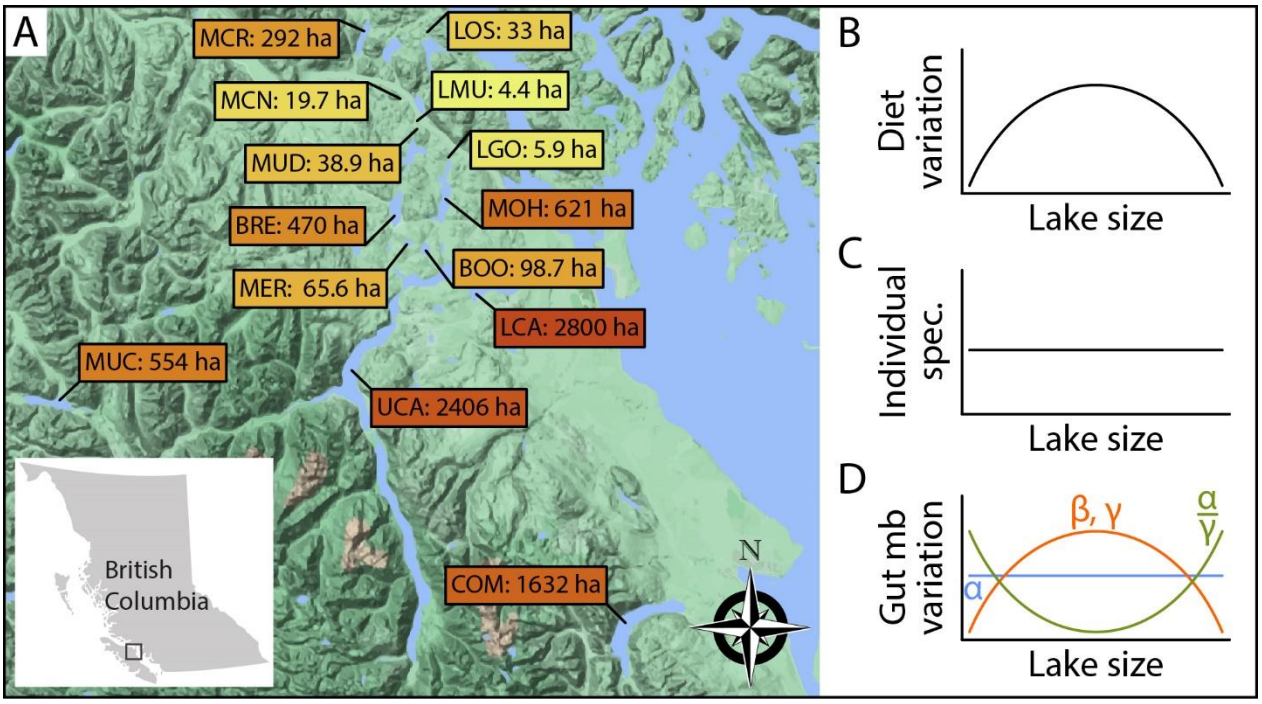

Figure 1: Map of Vancouver Island showing the location and size (shown in boxes, darker shading indicates larger lake size) of the 14 lakes included in this study (A). A previous study by Bolnick et al. (2020) showed that diet variation on the population level is highest in intermediate-sized lakes (B), but individual diet specialization is not associated with lake size (C). Based on these previous results, we predict that gut microbiota divergence (beta diversity), uniqueness (alpha diversity/gamma diversity ratio, note that smaller values indicate higher uniqueness), and diversity on the population level (gamma diversity), but not the individual level (alpha diversity), are greatest in intermediate-sized lakes (D).

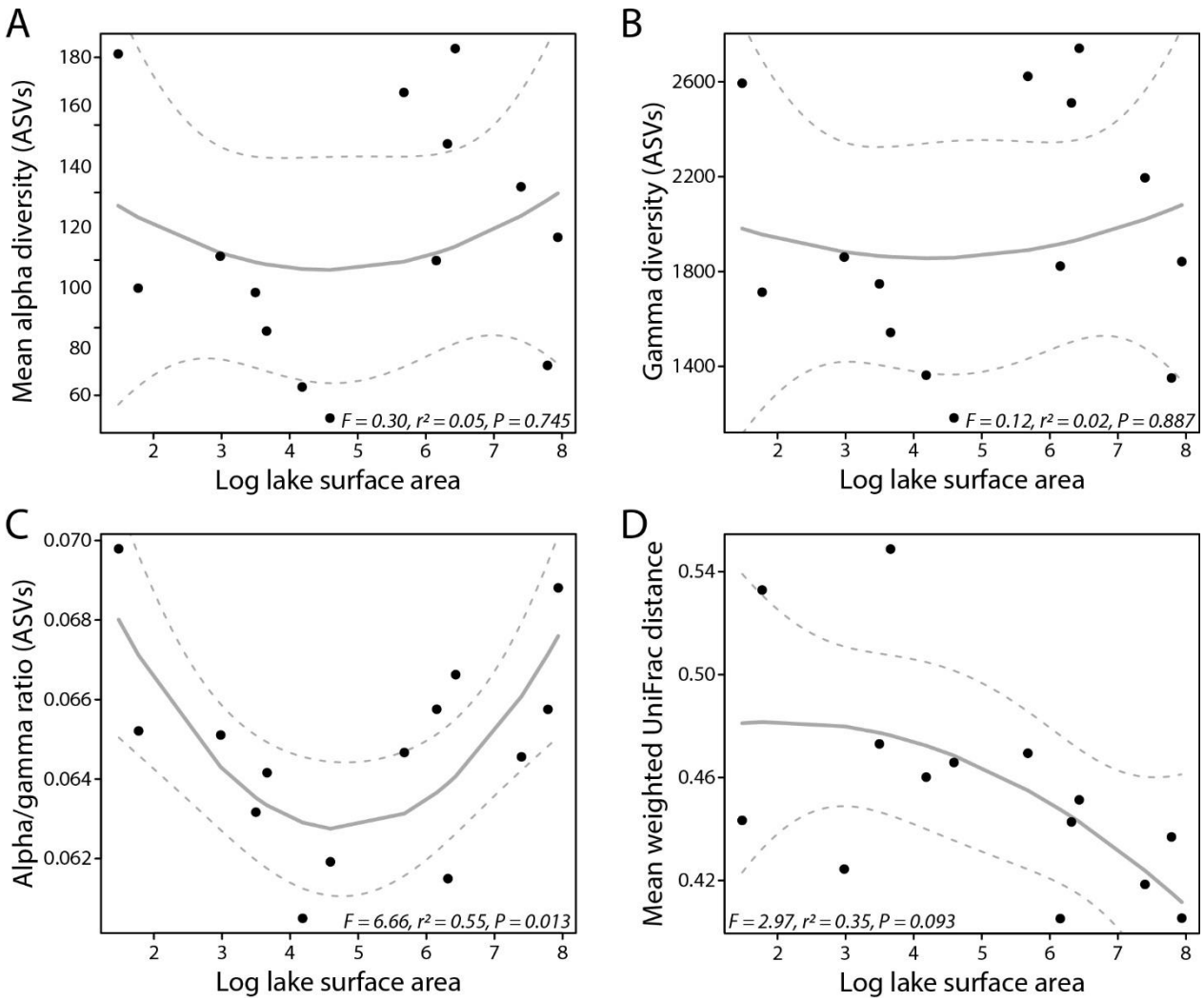

Figure 2: Association of gut microbiota diversity (A&B), uniqueness (C), and divergence (D) across populations from lakes of varying sizes. Neither mean alpha diversity (A) nor gamma diversity (B) showed increased values in intermediate-sized lake (i.e., lakes with greatest diet diversity) based on ASV richness. Alpha diversity/gamma diversity ratio was significantly lower in intermediate-sized lakes (C), suggesting greater gut microbiota divergence among individuals of these populations. There was suggestive evidence of a similar pattern for one beta diversity measure, weighted UniFrac (D), but not for Bray-Curtis dissimilarity or unweighted UniFrac (Figure S2). Solid lines show regression curves from quadratic models and dashed lines indicate 95% confidence intervals.