UNIVERSITY OF CALIFORNIA SAN DIEGO

Interactions between gut microbiome diversity, morphology, and trophic ecology in threespine stickleback fish (*Gasterosteus aculeatus*)

A Thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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The Thesis of Emma M. Kurstjens is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2023

DEDICATION

Dedicated to my mother, Lorna Kurstjens-Steytler, and her insistence on my finishing this thesis if only so she could be a published artist.

EPIGRAPH

Otto: What's this one's name? Well, not Wanda, anyway. I'm going to call him lunch. Hello Lunch! Hello!

A SLURP. A PAUSE. A SCOWL.

Otto: Eww. Avoid the green ones. They're not ripe yet.

Kevin Kline in *A Fish Called Wanda*

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ABSTRACT OF THE THESIS

Interactions between gut microbiome diversity, morphology, and trophic ecology in threespine stickleback fish (*Gasterosteus aculeatus*)

by

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Master of Science in Biology

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Professor Diana Rennison, Chair

Biotic interactions, including those on the microscopic level, are important contributors to adaptive evolution. This includes the host- associated microbiome, which is crucial for many aspects of their hosts' biology. However, the role of the microbiome in their hosts' adaptation to ecological niches remains largely unknown. Threespine stickleback represent a good model system to address this question. Notably, there is substantial variation in freshwater stickleback trophic ecology associated with lake size; populations in small lakes mainly feed on littoral invertebrates from the lake sediment (benthic prey) and populations in large lakes mainly feed on pelagic zooplankton (limnetic prey). Populations from

intermediate-sized lakes feed on a mix of benthic and limnetic prey. The evolution of trophic divergence along the benthic-limnetic axis as a result of repeated freshwater colonization allows the study of gut microbiome dynamics in response to varying environmental conditions. The goal of this project is to analyze the relationships between trophic ecology and morphological traits, as well as gut microbiome diversity, in order to examine patterns in the gut microbiome which might reflect niche specialization by threespine stickleback. Specifically, we hypothesized that populations from intermediate-sized lakes have the highest degree of gut microbiome diversity. Though two morphological traits correlate with the trophic ecology of lake size, there was minimal pattern in variation between gut microbiome diversity measures and the trophic ecology proxy or morphological data. Further study may improve our understanding of how a host, its gut microbiome, and the environment interact during the adaptation to different ecological niches.

INTRODUCTION

Natural selection is an integral force of change that acts upon all life forms. It drives evolution so that phenotypes that provide a competitive advantage proliferate with greater success into future generations (Kingsolver & Huey 2003). This boost in reproductive fitness due to advantageous traits within a particular ecological niche results in niche specialization of the species over the course of generations. This selective specialization can be the result of both biotic and abiotic factors. For example, a recent study of North American birds found that increasing global temperatures correlated with 80 of the 105 investigated species experiencing a reduction in body size over the 30-year period; this was proposed to be a mechanism to reduce energy expended to maintain homeostasis (Youngflesh 2022). Niche specialization also occurs in response to biotic interactions, such as interspecies competition or predation. Biotic interactions include infamous examples such as interspecies predation resulting in camouflage, exemplified in polymorphic prawns (*Hippolyte obliquimanus*) (Duarte et al. 2018; Stevens 2007). Impactful biotic interactions may also occur on a microscopic scale- but interactions with microbes are more poorly explored, and less understood.

Microbes can apply significant biotic pressures on macroscopic organisms. This is especially true of the bacteria that inhabit an organism, which are defined as the host's microbiome (Berg et al., 2020). These microbes are important for many of their host's physiological processes; for example, they are inovled in host immune responses (Christensen & Brüggemann 2013). In turn host factors have great influence on the continued survival of its passenger bacteria. This interdependence- along with exogenous genetic material, a variety of other microscopic life, and the multitude of organic molecules that microbes release to interact with their environment- is what shapes the microbiome (Berg et al., 2020). It has been

hypothesized that when the host evolves to fit an ecological niche, its microbiome will coevolve alongside it, each acting as a biotic pressure on the other in the course of evolution (Davenport et al. 2017; Koskella & Bergelson 2020; Rudman et al. 2019). The phenotypic traits that develop in the host as a consequence of these reciprocal interactions, as well as other jointly experienced selective pressures, could shape microbiome structure and function (Henry et al. 2021).

The gut microbiome in particular has recently become of intense interest to the scientific community (Härer et al. 2020; Neu et al 2021; Trevelline et al. 2019). Interrelations between host and gut microbiome mean that host-related factors that underlie niche specialization may correlate with gut microbiome diversity. One such factor is morphology of the host. For example, a study found that koalas (*Phascolarctos cinereus*) have a notably longer hindgut, which along with gut bacteria from the Pasteurellaceae family- which express enzymes capable of processing tannins- allow koalas to survive on their exclusive diet of nutrient-poor eucalyptus leaves (Chong et al. 2019, Osawa et al. 1995). Another important factor that influences the host and thus its microbiome is trophic ecology- namely, an organism's diet. An extreme example of this is the gut microbiome of the common vampire bat (*Desmodus rotundus*). Zepeda-Mendoza et. al discovered collaboration between the bat's own proteome and its gut microbiome; both express iron sequestering properties that help process the host's sanguivorous diet to sustain each participant (Zepeda-Mendoza et. al, 2018). These interspecies interactions reflect the close connection between hosts and their microbiomes and their potential effects on the evolution of each. Given these observations in unrelated taxa, we could predict that more generally there may often be covariance between microbiome variation and host morphology might be strongest for traits involved in feeding. Studying the niche evolution of a higher-level organism without taking into account the dependency it has on its microbiome is to neglect half the story.

A prime organism to directly investigate the relationships between microbiome, morphology, and trophic ecology is the threespine stickleback fish (*Gasterosteus aculaeatus*). Populations of threespine stickleback independently colonized post glacial lakes throughout the Northern Hemisphere and have been evolving in parallel over the last 13,000 years (Bell & Foster, 1994). This is ideal, as different levels of phylogenetic divergence among host lineages may be a confounding factor in gut microbiome comparison studies (Brooks et al., 2017). Parallel evolution of stickleback within ecologically equivalent conditions has also given rise to a spectrum of well-established morphologies suited to the niches within these lakes. Benthic ecotype fish are characterized by their generally larger body shape and wide mouths to accommodate their shallow-feeding lifestyle; the limnetic ecotype is much narrower and lives in open water, feeding on plankton (McPhail 1993; Bell & Foster 1994). The prevalence of this morphological spectrum of niche specialization provides a pre-established dimension for analysis. Further, Rennison et al. (2019) found evidence of parallel gut microbiome evolution in three lakes which contain sympatric pairs of benthic and limnetic stickleback, and it stands to reason that this may continue to occur to some degree in less distinctive ecotypes found across solitary lakes.

The goal of this study was to analyze the relationships between trophic ecology, morphological traits and gut microbiome diversity to determine the factors associated with niche specialization in threespine stickleback. Previously, Bolnick and Ballare (2020) found a strong negative linear relationship between lake surface area and mean proportion of benthic diet, an indication of trophic specialization, in stickleback across 21 lakes on Vancouver Island, Canada. This observation suggests that lakes with a greater surface area likely contain a greater proportion of limnetic fish and smaller lakes contain relatively more benthic fish. This

observation also establishes lake size as a proxy for trophic ecology. Another finding from the same study was that mean pairwise diet differences among individuals within lake populations are maximized in intermediate sized lakes – which is important because it indicates that there may greater gut microbiome diversity within these lakes , a consequence of variation in diet items. For this study we surveyed wild caught fish from fourteen of the lakes included in Bolnick and Ballare (2020), all of which are located on Vancouver Island, Canada (Figure 1). The previously established relationship between gut microbiome and trophic ecology of the host (Härer et al. 2020; Rennison et al. 2019; Zepeda-Mendoza et. al, 2018) leads to the formation of several hypotheses: firstly, that certain traits will correlate with gut microbiome diversity, either across or within lake populations, as a reflection of their coevolution. Those traits are expected to be related to foraging, and include mouth width, lower jaw length, eye diameter, head length, and epaxial length. Secondly, we hypothesize that populations from lakes of intermediate size, having the most diverse diet difference between individuals, will have the most diverse gut microbiomes.

METHODS

Field collection

Threespine stickleback specimens were collected from fourteen lakes of varying size on Vancouver Island, British Columbia, Canada in spring 2020 and 2021 (Figure 1.1). Roughly equal numbers of adult male and female fish (5-15 females, 11-14 males) from each of the lakes were included in the analysis (Table 1). These individuals were euthanized with an overdose of MS-222 (500 mg/L) and kept frozen at -70 °C for later examination.

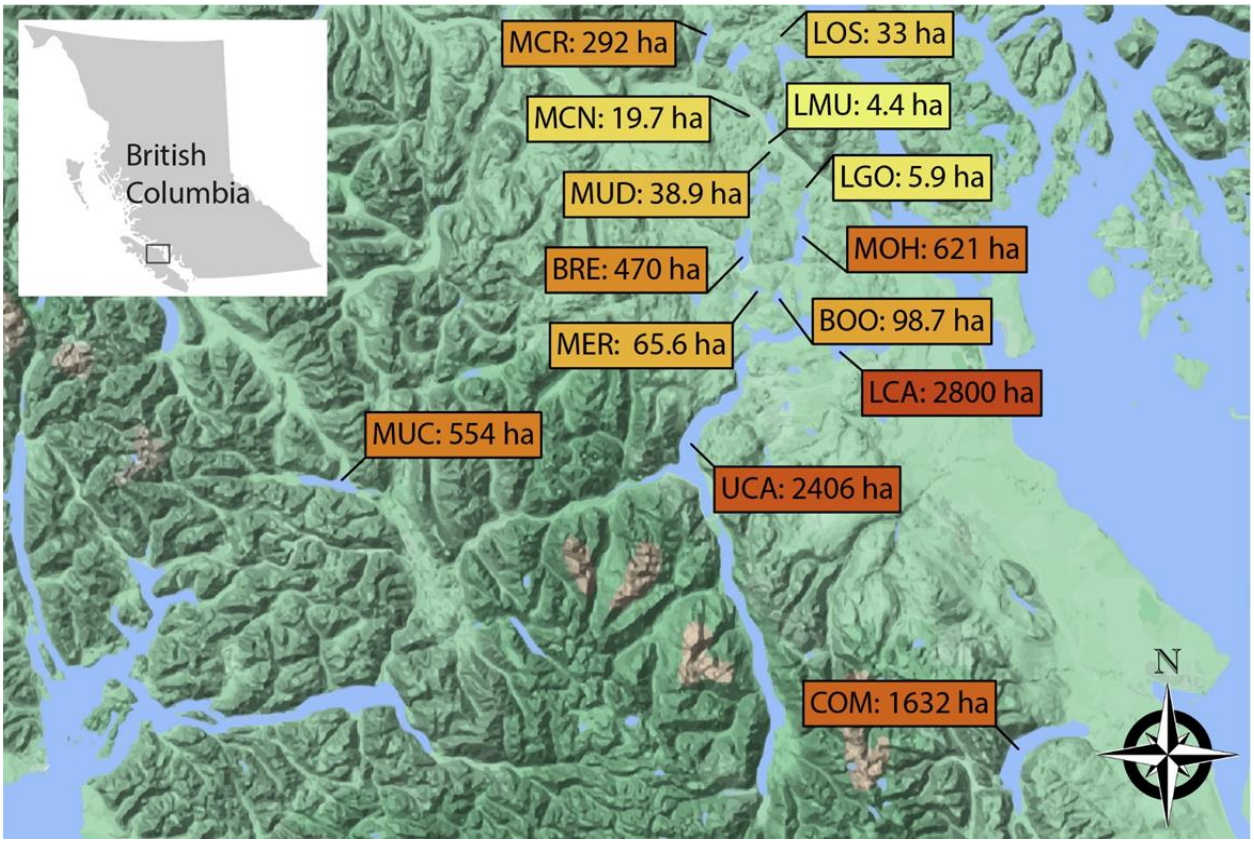

Figure 1.1: A subset of a geographical map of Vancouver Island, British Columbia. Lakes are labeled with an abbreviation of their name and surface area in hectares.

Table 1.1: A list of lakes included in this study, along with their surface area in hectares, the watershed that they are part of, and the numbers of male and female fish from each lake included in our analyses.

Lake	**Watershed**	**Surface area (hectares)**	**Sex: Female/ Male**	
Little Mud	Amor	4.40	14	14
Little Goose	Mohun	5.90	13	13
McNair	Amor	19.70	11	11
Lower Stella	Pye	33.00	12	12
Mud	Amor	38.90	13	14
Merrill	Campbell	65.62	12	14
Boot	Campbell	98.67	13	13
McCreight	Amor	292.00	5	11
Brewster	Campbell	470.00	14	14
Muchalat	Gold	554.00	14	13
Mohun	Mohun	620.90	13	13
Comox	Comox	1631.70	15	11
Upper Campbell	Campbell	2406.00	14	13
Lower Campbell	Campbell	2800.00	13	14

Phenotyping Specimens

For preservation, specimens were fixed in a 10% formalin solution for two weeks. Each fish was stained with alizarin red to highlight bony morphological features (e.g. Figure 2.1). This was done by first rinsing the fish in water for 24 hours to remove the formalin, followed by staining in a solution of alizarin red and potassium hydroxide for 48 hours with a final 24 hour water rinse and long term storage in 40% isopropanol. Twelve morphological traits were measured in triplicate, with the three measurements averaged. Body length, body depth, pelvic spine length, lengths of the first and second dorsal spines, mouth width, lateral plate number, eye diameter, and caudal-peduncle width were measured using calipers (Figure 2.1, 1-9

respectively). Head length, epaxial length, and lower jaw length were measured in Image J using high- resolution photographs (Figure 2.2).

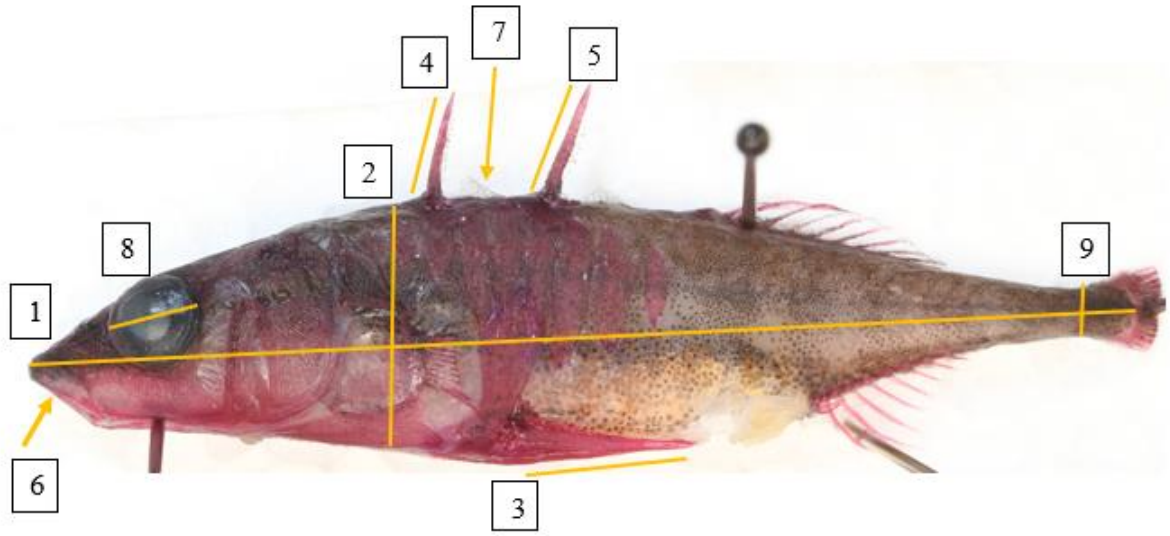

Figure 2.1: Features measured with calipers.

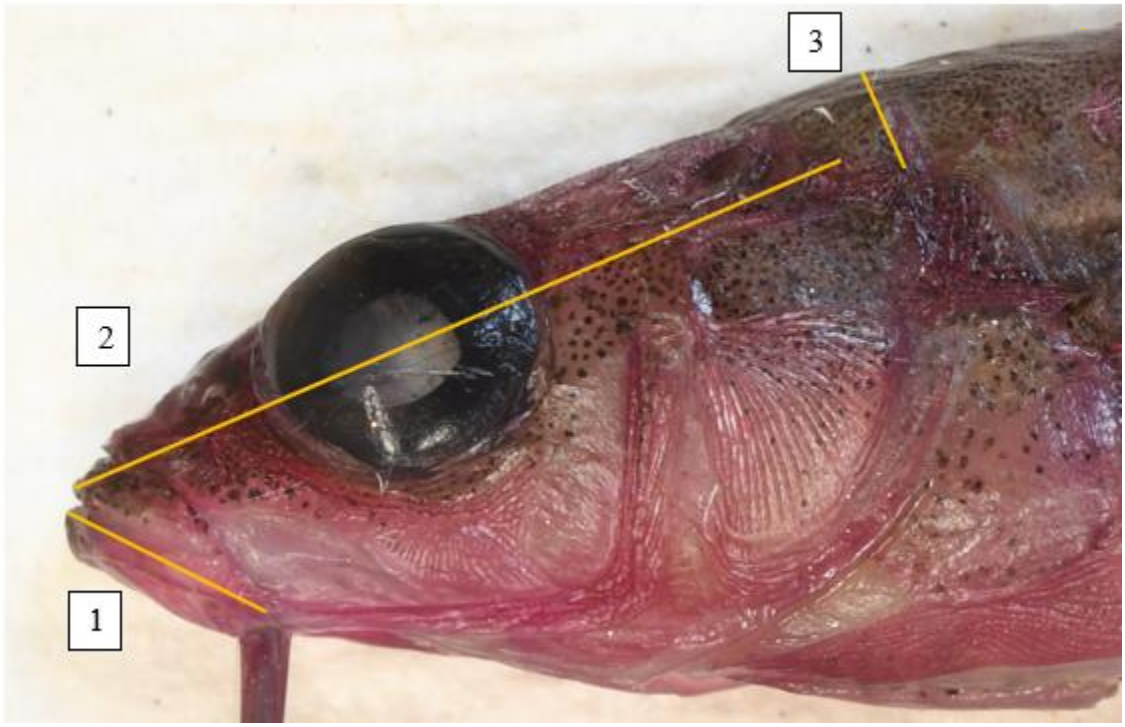

Figure 2.2: Features measured with Image J.

There was a significant positive correlation between body length and eleven of the traits (lateral plate count did not scale). Thus, size correction was performed using the following equation:

$$y_i = x_i - \beta(L_i - L)\ ,$$

where y_i is the size-adjusted morphological trait, x_i is the original trait, β is the regression coefficient of the un-adjusted trait values on standard length, L_i is the standard length of the individual and L is the average sample length across all lakes (47 mm). The size corrected measurements were then used in all subsequent analyses.

DNA extraction and library preparation

To analyze the gut microbiome of the specimens, the whole digestive tract of each defrosted sample was removed using sterile equipment and frozen at -70 °C until time of DNA extraction. DNA was extracted using QIAGEN's PowerSoil Pro Kit according to the manufacturer's protocol. Once extraction was complete, PCR was performed on the 16S ribosomal RNA gene with barcoded 515F x 806R primers (see the protocol published by Kozich et al. for primer sequences).

PCR amplifications were performed in triplicate with a 10 µl reaction volume using Platinum II Hot Start PCR Master Mix and pooled. The PCR cycle consisted of a denaturation step lasting 60 s at 98 °C, 35 amplification cycles lasting 10 s at 98 °C, 20 s at 56 °C and 60 s at 72 °C, and a final elongation step at 72 °C for 10 min. Gel electrophoresis was performed on a 2% agarose gel for all samples to visually check for amplification specificity. DNA concentrations of pooled PCR products were again measured on a Qubit 4 Fluorometer, and samples were pooled in an equimolar manner to construct a single library. This library was sent to the UC Davis Genome Center to be purified by bead clean-up and run on a Bioanalzyer to check DNA quality. The final

library was sequenced on one lane of the Illumina MiSeq 600 (PE300) platform.

Sequencing reads were processed in QIIME2. In order to obtain amplicon sequencing variants (ASVs), we used the QIIME2 plugin dada2 to check the quality of our sequence data, correct sequencing reads, and filter chimeric sequences (Callahan, et al. 2016). We constructed a phylogenetic tree of the bacterial lineages present in our samples with FastTree 2.1.3 (Price, et al. 2010) as implemented in QIIME2, which is necessary to calculate phylogenetic distance matrices for the two UniFrac metrics used later in gut microbiome analysis. We assigned bacterial taxonomy against the SILVA 132 ribosomal RNA (rRNA) database at a 99% similarity threshold (Quast, et al. 2013). ASVs that either only occurred in one sample, could not be assigned below the phylum level, or belonged to either chloroplasts, mitochondria, cyanobacteria, or archaea were filtered from the data set.

Gut microbiome analysis

There are multiple methods of quantification that may be used when measuring diversity. Alpha diversity measures species richness within a sample (e.g., the gut of an individual host), which can be a raw count of the number of species present or can account for relative abundance of species within said sample; beta diversity is defined as relative differences measured between samples (Whittaker 1972).

For alpha diversity, the number of ASVs and Shannon's entropy scores were used to quantify the alpha diversity of each individual's gut microbiome. ASV counts identify each unique bacterial lineage collected from high- throughput sequencing of an individual's gut microbiome. Shannon entropy score takes into consideration both the total number of bacterial lineages and their relative abundance to construct a more detailed understanding of the bacterial community. Once obtained, these metrics were used in statistical analyses.

Beta diversity of the gut microbiome was quantified as unweighted Unifrac, weighted Unifrac, and Bray-Curtis dissimilarity. The Unifrac calculations performed use the phylogenetic trees constructed in QIIME2 to find the fraction of unique evolutionary branching each microbial sample's tree contains, pairwise to each other sample (Lozupone 2011). While unweighted Unifrac only considers presence-absence of bacterial lineages, weighted Unifrac further takes into account their abundances in the sample (Lozupone 2007) Bray Curtis dissimilarity was calculated in order to show the number of different bacterial lineages found in each sample relative to each other sample. Unweighted Unifrac, weighted Unifrac, and Bray-Curtis dissimilarity calculations were performed such that distance matrices with pairwise distances between all specimens across all lakes were constructed.

Statistical Analysis

All statistical tests were performed in R (2022.07.02) using RStudio. Linear and quadratic models were used to assess associations between the size corrected morphological trait measurements and the natural log of lake size, which we used as a proxy for trophic ecology based on Bolnick & Ballare (2020). The standard deviation of the size corrected measurements was calculated for each trait within each lake to obtain information on the morphological variation within each population. Linear and quadratic models were then applied to those standard deviation values to investigate their relationship with lake size. Principal component analysis was conducted using all morphological measurements except body depth, pelvic spine, and 1st dorsal spine as they were overly correlated with other traits. This was used to see if particular populations of stickleback were grouped by lake size, as well as for later correlation analysis by PC scores.

The ASV numbers and Shannon entropy scores of individual fish were grouped by lake of origin; then, linear and quadratic models were used to assess the relationship between the alpha diversity measures and lake size. Spearman correlation tests were then performed within and across lakes between alpha diversity measures and morphological measurements to assess the relationships between morphology and alpha diversity. The resulting coefficients were represented in heat maps to show relative strength of these correlations and boxplots were used to display variation in correlation coefficients across lakes. Heatmaps were also constructed to show the correlation coefficients calculated from the PC1 and PC2 scores for morphological traits and alpha diversity measures within lakes.

Beta diversity measures of gut microbiome diversity were used in conjunction with distance matrices of morphological traits to test for a relationship between these factors. Mantel tests were performed between distance matrices of all qualifying samples' size-corrected traits and each metric of gut microbiome beta diversity- weighted UniFrac, unweighted UniFrac, and Bray-Curtis dissimilarity- to see if the differences in individuals' morphology within and across lakes covaries with their respective gut microbiome beta diversity measures. Correlation coefficients were also summarized as heat maps and boxplots to display the variation in Mantel test results.

RESULTS

Morphology and Trophic Ecology

To characterize the potential relationships between trophic ecology, morphology and gut microbiome diversity, it is important to assess first any interactions between the prior two components. Table 2 displays associations between mean size corrected morphological traits and the natural log of lake size, used as the trophic ecology proxy. Of the eleven traits tested, lower jaw length ($P = 0.0211$, $R^2 = 0.4191$) and lateral plate count ($P = 0.01326$, $R^2 = 0.4945$) were the only traits with a significant relationship with lake size, and only when fitted with a quadratic model. These scores are indicative of a higher average trait size in intermediate sized lakes.

Table 2.1: Table of linear and quadratic regression statistics derived from size-corrected morphological traits versus natural log of lake size. Significant relationships are highlighted in bold.

Traits	Linear Model Statistics				Quadratic Model Statistics			
	P value	Adj. Rsq	F	DF	P value	Adj. Rsq	F	DF
Head length	0.2842	0.01937	1.257	12	0.1697	0.1439	2.093	11
Epaxial length	0.218	0.0504	1.69	12	0.08967	0.2377	3.027	11
Lower jaw length	0.09426	0.1504	3.302	12	**0.02011**	**0.4191**	**5.69**	**11**
Body depth	0.8423	-0.07962	0.04131	12	0.8353	-0.1438	0.183	11
Pelvic spine	0.4687	-0.03504	0.5599	12	0.7398	-0.1188	0.3098	11
1st dorsal spine	0.8554	-0.08021	0.03466	12	0.9736	-0.1761	0.02682	11
2nd dorsal spine	0.6474	-0.06382	0.2201	12	0.8389	-0.1447	0.1785	11
Mouth width	0.843	-0.07965	0.04096	12	0.9707	-0.1754	0.02984	11
Lateral plate count	0.1599	0.0958	2.271	11	**0.01326**	**0.4945**	**6.871**	**10**
Eye diameter	0.8789	-0.08115	0.02422	12	0.9222	-0.1645	0.08164	11
Caudal peduncle length	0.9878	-0.08331	0.0002452	12	0.4202	-0.00946	0.9391	11

Based on Bolnick and Ballare (2020), our supposition was that variance in morphological traits would peak in intermediate size lakes due to the relatively equal proportion of benthic and limnetic habitat. This would lead to a quadratic fit of the data. However, neither a linear nor quadratic model was found to fit the projected pattern of variation, quantified as standard deviation, in trait dimensions seen across lake size (Table 3).

Table 2.2: Table of linear and quadratic regression statistics derived from size-corrected morphological traits' standard deviations versus natural log of lake size.

Traits	Linear Model Statistics				Quadratic Model Statistics			
	P value	Adj. Rsq	F	DF	P value	Adj Rsq	F	DF
Head length	0.6239	-0.06625	0.2544	11	0.617	-0.08953	0.507	10
Epaxial length	0.08636	0.1607	3.49	11	0.1191	0.1973	2.597	11
Lower jaw length	0.5144	-0.04406	0.4514	11	0.7701	-0.127	0.2675	11
Body depth	0.1641	0.08426	2.196	12	0.1094	0.2096	2.724	11
Pelvic spine	0.5964	-0.05726	0.2959	12	0.8641	-0.1509	0.148	11
1st dorsal spine	0.8902	-0.08154	0.01987	11	0.9329	-0.167	0.06995	11
2nd dorsal spine	0.5446	-0.04933	0.3888	12	0.2226	0.1007	1.728	11
Mouth width	0.9064	-0.08203	0.01443	12	0.6443	-0.09103	0.4577	11
Lateral plate count	0.6911	-0.07464	0.1665	11	0.8614	-0.1647	0.1515	10
Eye diameter	0.5085	-0.04647	0.4671	11	0.3785	0.0119	1.072	10
Caudal peduncle width	0.1631	0.08499	2.208	12	0.2496	0.08178	1.579	11

A PCA was conducted to visualize the distribution of individuals' morphological data, based on lake of origin (Figure 3.1). Body depth, pelvic spine, and 1st dorsal spine were excluded, as they were overly correlated with other traits. This analysis did not find clustering by lake size along either the PC 1 or PC 2 axes, which explained 35.5% and 24.9% of the variation in the data, respectively. Interestingly, the clustering pattern along PC2 appears to be related to watershed; the upper grouping includes eight lakes from the Campbell River and Amor watersheds, whilst the bottom six lakes are part of the Comox, Campbell, Gold, Mohun, and Pye watersheds.

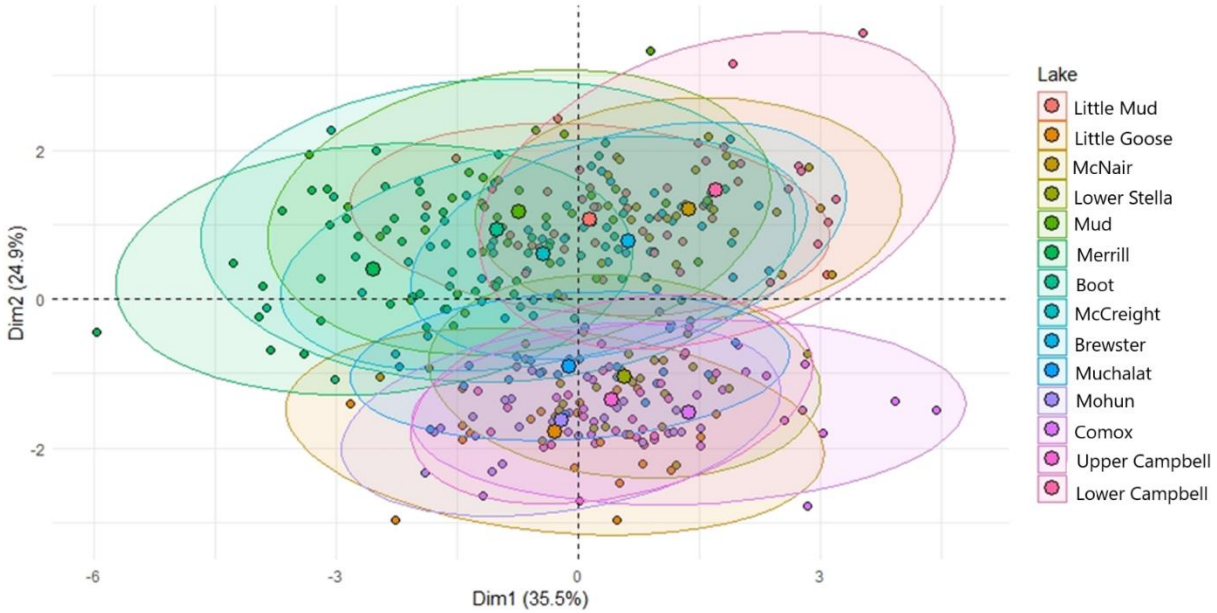

Figure 3.1: Principal component analysis of individuals' morphological traits, with grouping based on lake of origin.

Alpha Diversity

Alpha diversity was the first measure of gut microbiome diversity assessed against the trophic ecology proxy and morphological measurements. Two metrics were used to quantify alpha diversity: number of ASVs and Shannon entropy scores. A small number of samples was removed (between 1-6 per lake) as some stickleback that were phenotyped did not give adequate gut microbiome data. No significant linear or quadratic relationship was found between lake size and mean number of ASVs per individual (Figure 4.1, Table 3). Linear and quadratic models were also applied to Shannon entropy scores with similar negligible effect (Figure 4.2, Table 4).

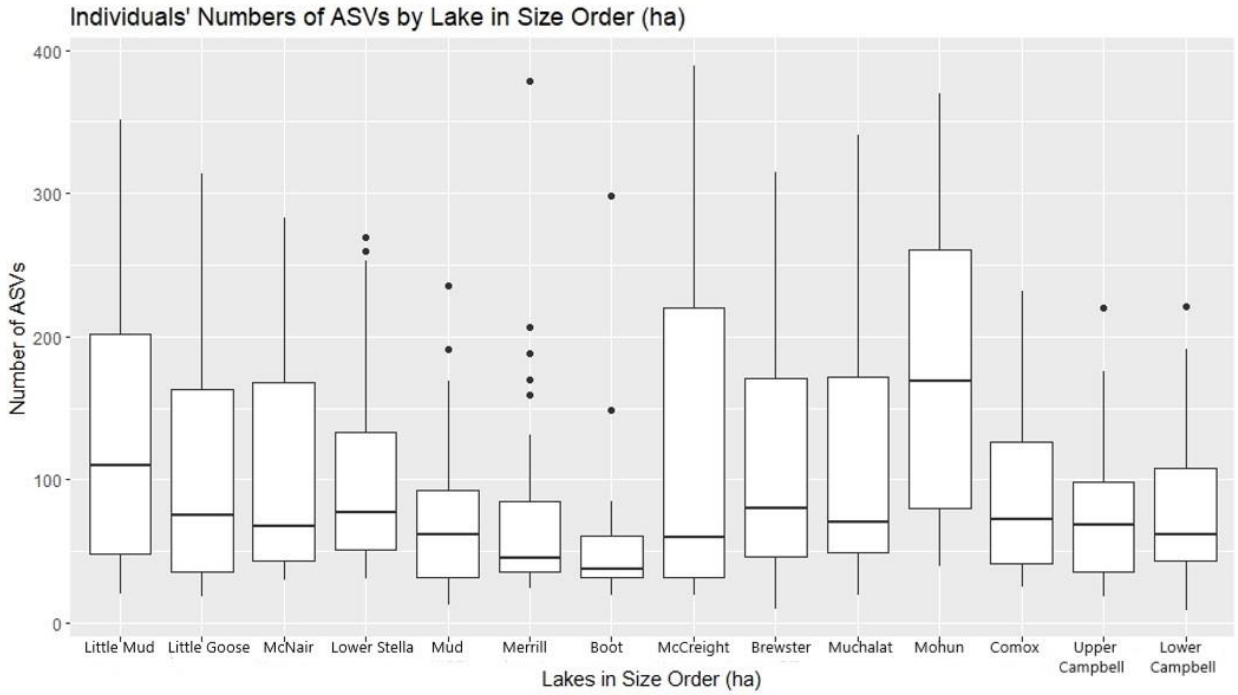

Figure 4.1: Variation in ASV counts (alpha diversity) by lake of origin with lakes ordered by increasing surface area.

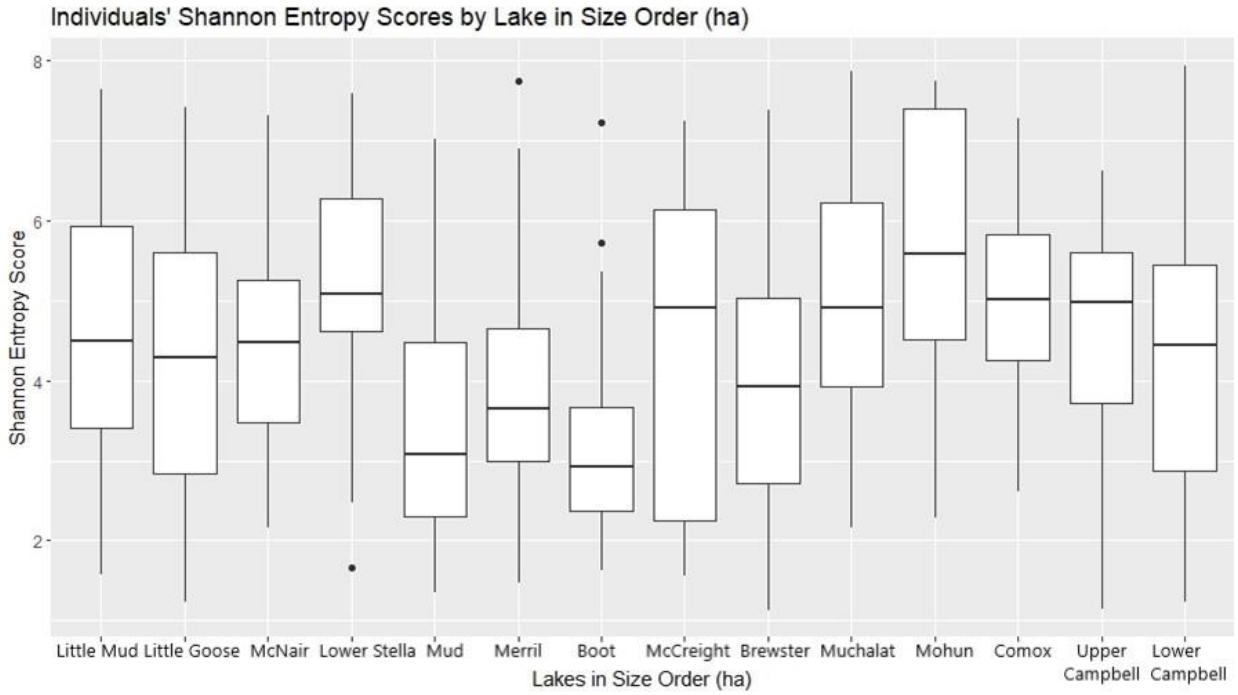

Figure 4.2: Variation in Shannon's entropy by lake of origin with lakes ordered by increasing surface area.

Table 3.1: Table of linear and quadratic regression statistics derived from ASV number and Shannon entropy scores versus natural log of lake size.

Traits	Linear Model Statistics				Quadratic Model Statistics			
	P value	Adj. Rsq	F	DF	P value	Adj Rsq	F	DF
ASV number	0.9186	-0.08235	0.01089	12	0.7055	-0.1092	0.3601	11
Shannon Entropy Score	0.6076	-0.0588	0.278	12	0.7403	-0.1189	0.3091	11

Statistical tests were performed to assess whether morphological variation is associated with gut microbiome alpha diversity, such that traits may act as predictors of gut microbiome composition. Correlation coefficients for the two alpha diversity metrics and trait morphology, are summarized in two heat maps (Figures 5.1 and 6.1). Between morphology traits and ASV number the correlations ranged from -0.52 to 0.69 (Figure 5.1). For Shannon entropy scores the correlation coeffiients with morphology ranged from -0.59 to 0.60 (Figure 6.1). Overall, neither alpha diversity metric showed consistent correlation (differed significantly from zero) with the morphological traits surveyed (Figures 5.2 and 6.2). Correlation coefficients were also calculated for PC 1 and PC 2 of morphology for both ASV count and Shannon Entropy (Figures 7.1 and 7.2). These analyses also did not identify a consistent relationship across lakes, with correlation coefficient ranges between -0.45 to 0.29 and -0.22 to 0.28, respectively.

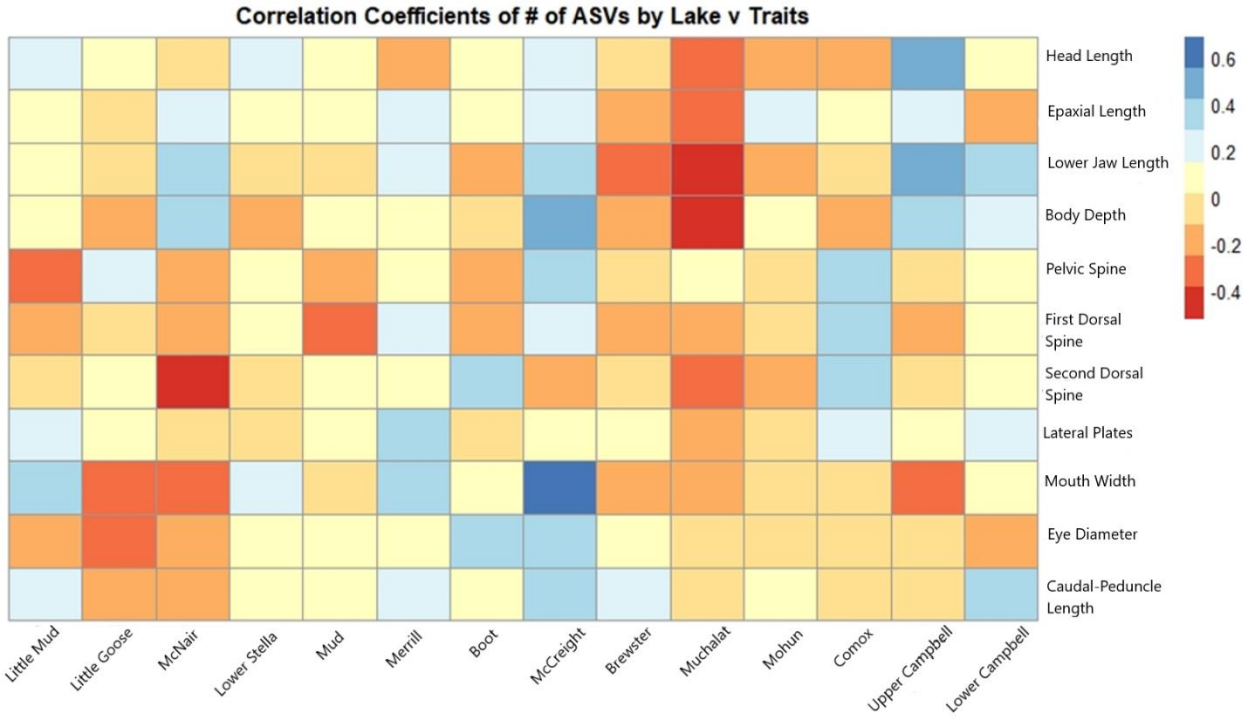

Figure 5.1: Heatmap showing the strength of correlation coefficients generated between each trait on the x axis and ASV counts within each lake. Lakes are in order of increasing surface area on the y axis.

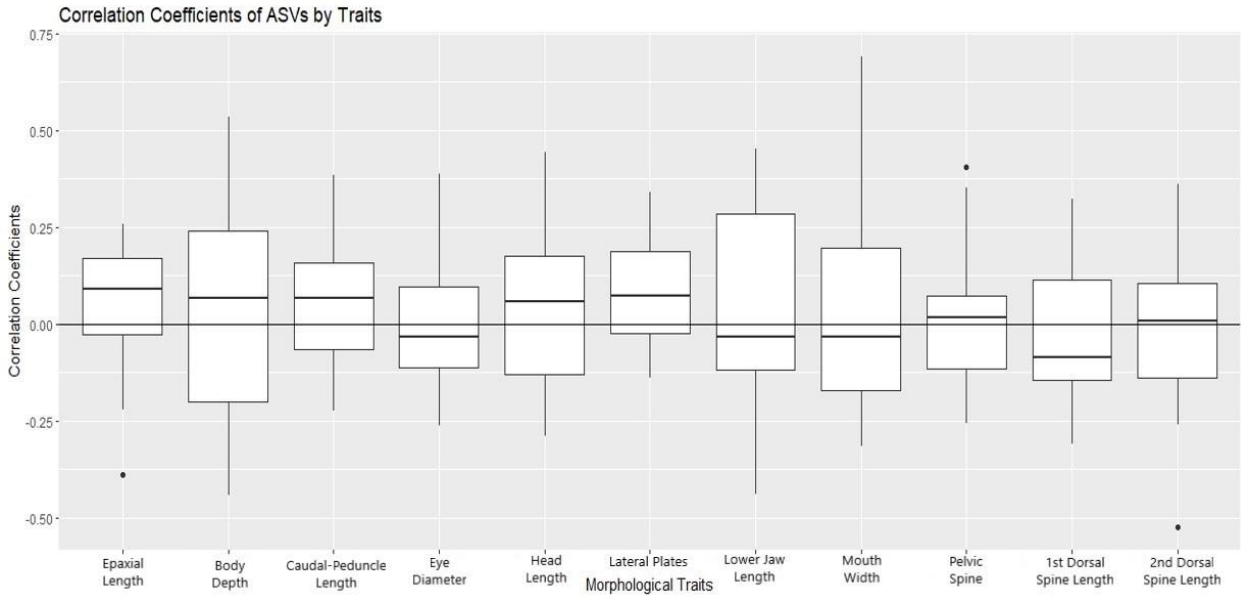

Figure 5.2: Variation in correlation coefficients between trait values and ASV counts.

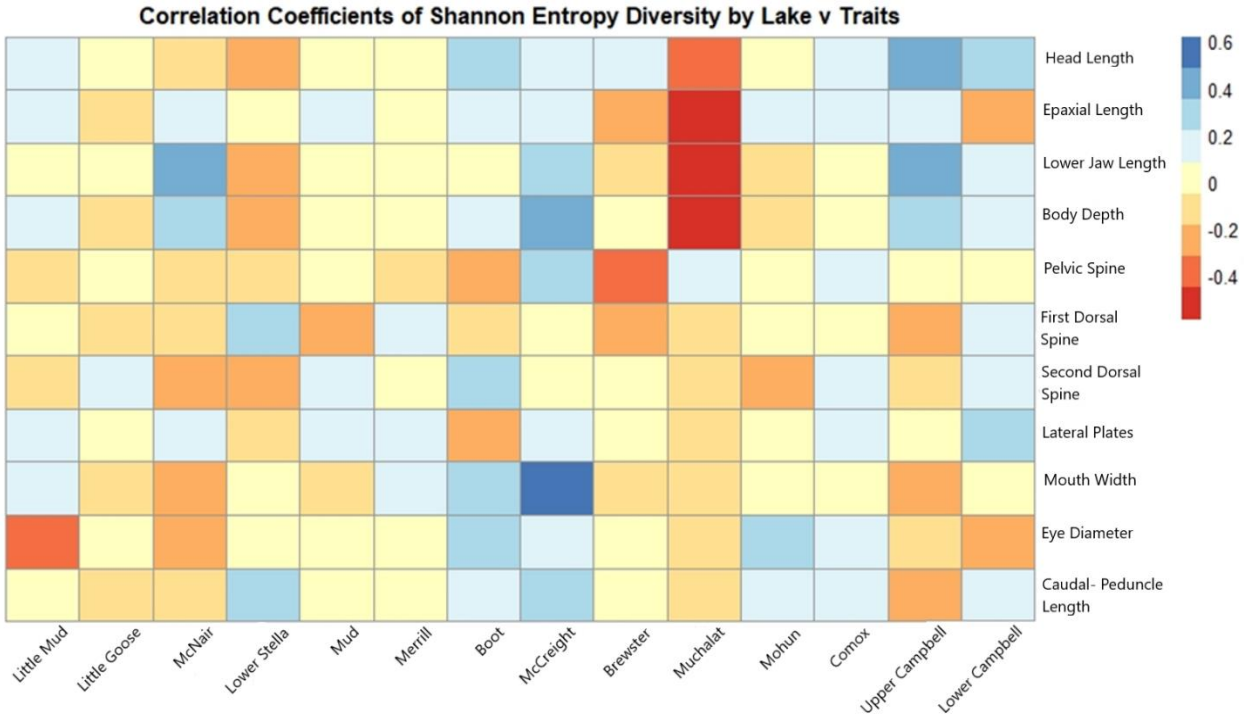

Figure 6.1: Heatmap showing the strength of correlation coefficients generated between each trait on the x axis and Shannon entropy scores within each lake. Lakes are in order of increasing surface area on the y axis.

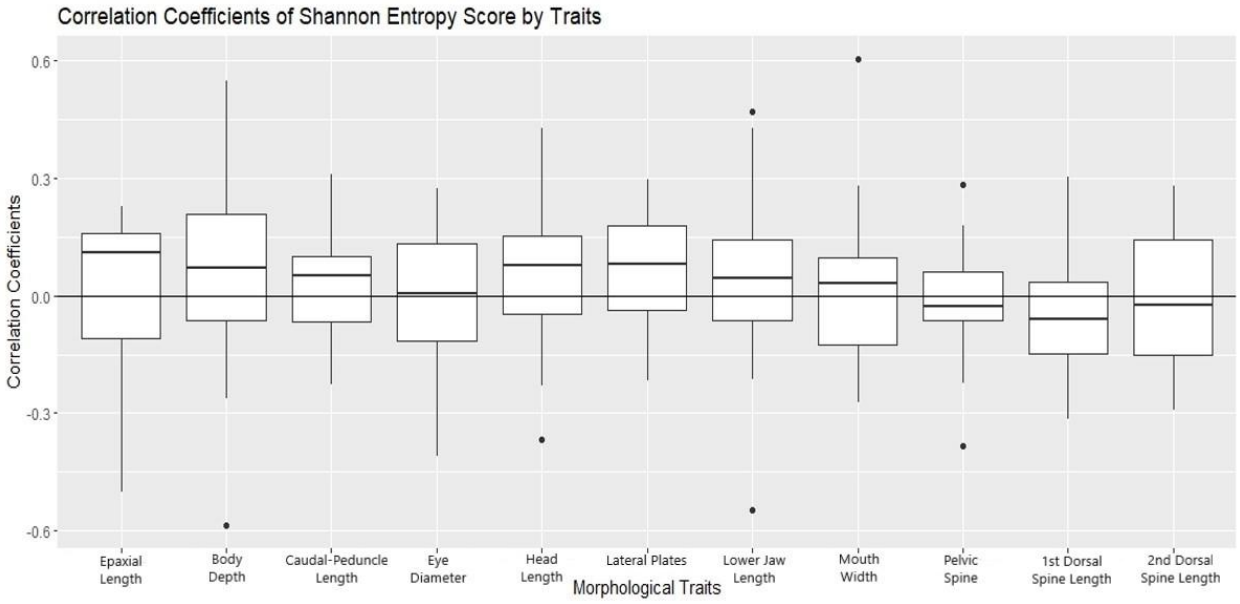

Figure 6.2: Variation in correlation coefficients generated between each trait and Shannon entropy scores.

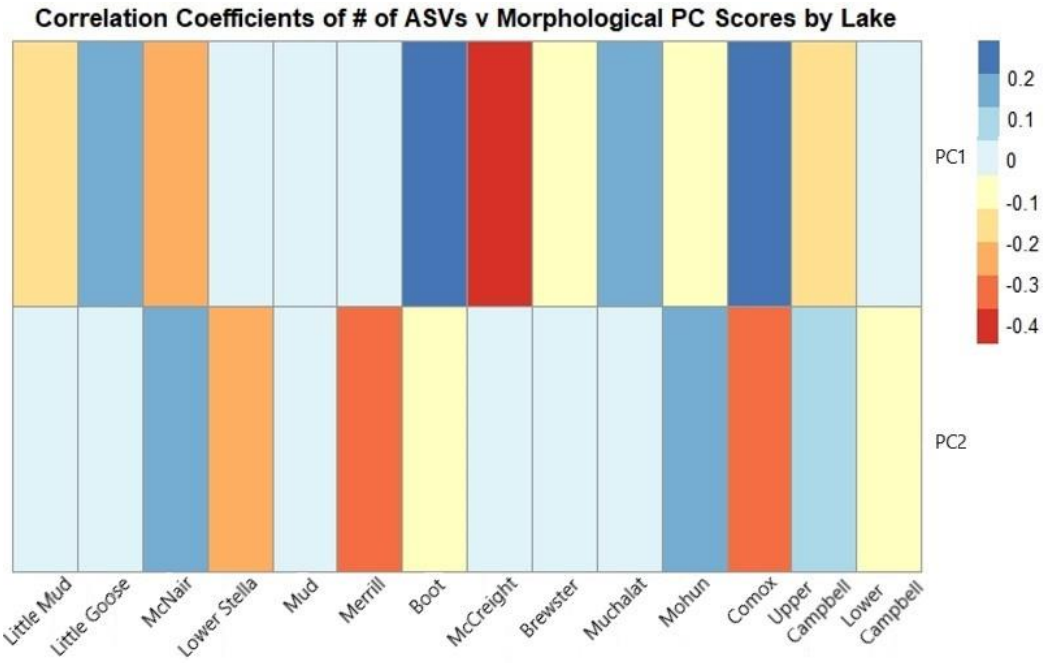

Figure 7.1: Heatmap showing the strength of correlation coefficients generated between PC scores on the x axis and ASV counts within each lake. Lakes are in order of increasing surface area on the y axis.

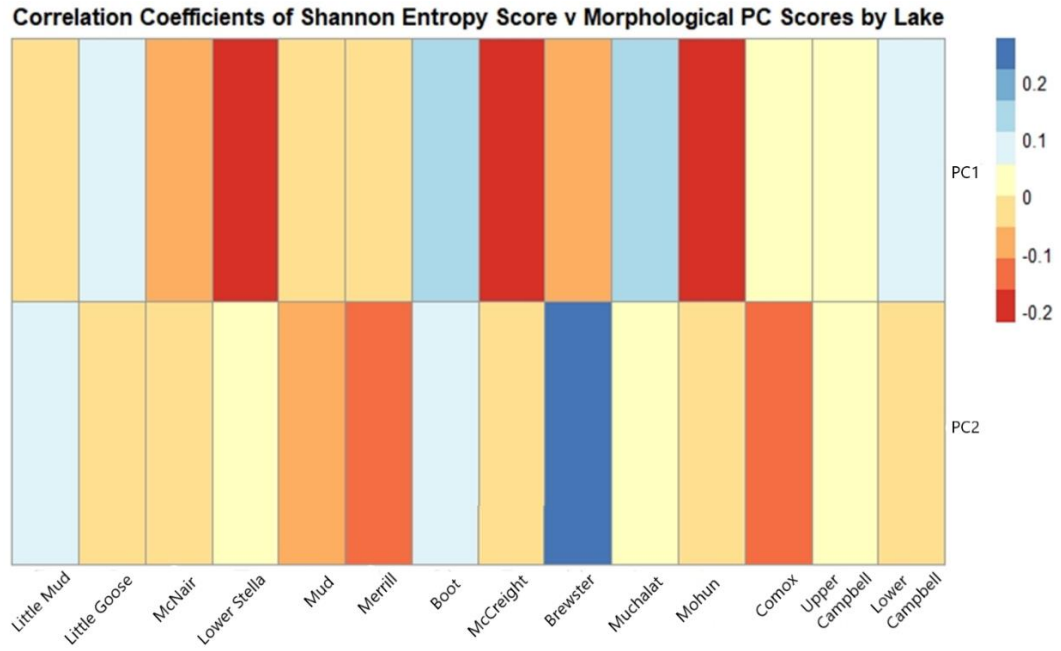

Figure 7.2: Heatmap showing the strength of correlation coefficients generated between PC scores on the x axis and Shannon entropy scores within each lake. Lakes are in order of increasing surface area on the y axis.

Beta Diversity

Beta diversity measures of gut microbiome diversity were used in conjunction with distance matrices of morphological traits to test for relationships between these factors, both within lake and across lakes. Firstly, Mantel tests were performed between distance matrices of all traits (excluding lateral plates) across all lakes and each metric of gut microbiome beta diversity- weighted UniFrac, unweighted UniFrac, and Bray-Curtis dissimilarity. None of the morphological traits showed any significant relationship with any of the three beta diversity metrics, as indicated by the near-zero test statistics (Table 5).

Table 4.1: Table of Mantel test R statistics from comparing distance matrices of weighted Unifrac, unweighted Unifrac, and Bray-Curtis dissimilarity against distance matrices of measured traits.

Trait	Weighted Unifrac	Unweighted Unifrac	Bray Curtis
Head length	0.02854933	0.01889203	0.01526437
Epaxial length	0.007454868	0.001702546	0.03012273
Lower jaw length	0.06606946	0.0151515	0.02059747
Body depth	-0.01603721	0.03367867	0.03407619
Pelvic spine	0.08637268	0.008388428	0.06527353
1st dorsal spine	0.07413875	-0.005680278	0.05121747
2nd dorsal spine	0.0888086	-0.02451693	0.04495746
Mouth width	0.06148855	0.09324966	0.1323084
Eye diameter	-0.04049382	-0.01675387	-0.01787637
Caudal peduncle length	0.01857685	-0.01227622	0.01797566

Next, distance matrices were constructed for individual lake populations' traits and set against the corresponding beta diversity matrices to obtain the desired Mantel test results. The weighted UniFrac heatmap legend on the upper right shows that the R statistics range from -0.2548258 to 0.2502773 (Figure 8.1). Further, there is no consistent relationship between the morphological traits on the y axis and their respective weighted UniFrac score across the size ordered lakes on the x axis (Figure 8.2). This pattern is also observed for unweighted UniFrac scores, with a range of Mantel R statistics from -0.1594695 to 0.383804 (Figures 9.1 & 9.2). The heatmap of Bray-Curtis dissimilarity versus lake population morphology R statistics also reflects

weak relationships between gut microbiome beta diversity and measured traits, with the range of R statistics between -0.2104881to 0.2037448 (Figures 10.1 & 10.2).

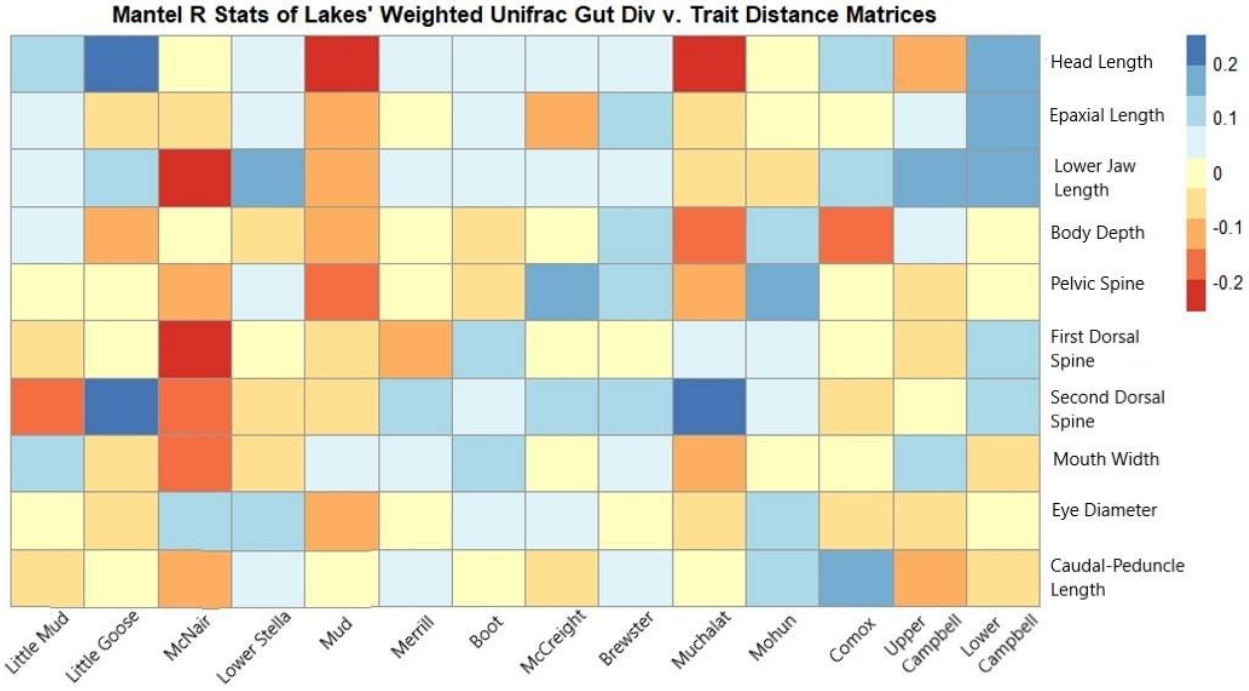

Figure 8.1: Heatmap showing the strength of Mantel R statistics generated between a distance matrix of each trait on the x axis and weighted Unifrac within each lake. Lakes are in order of increasing surface area on the y axis.

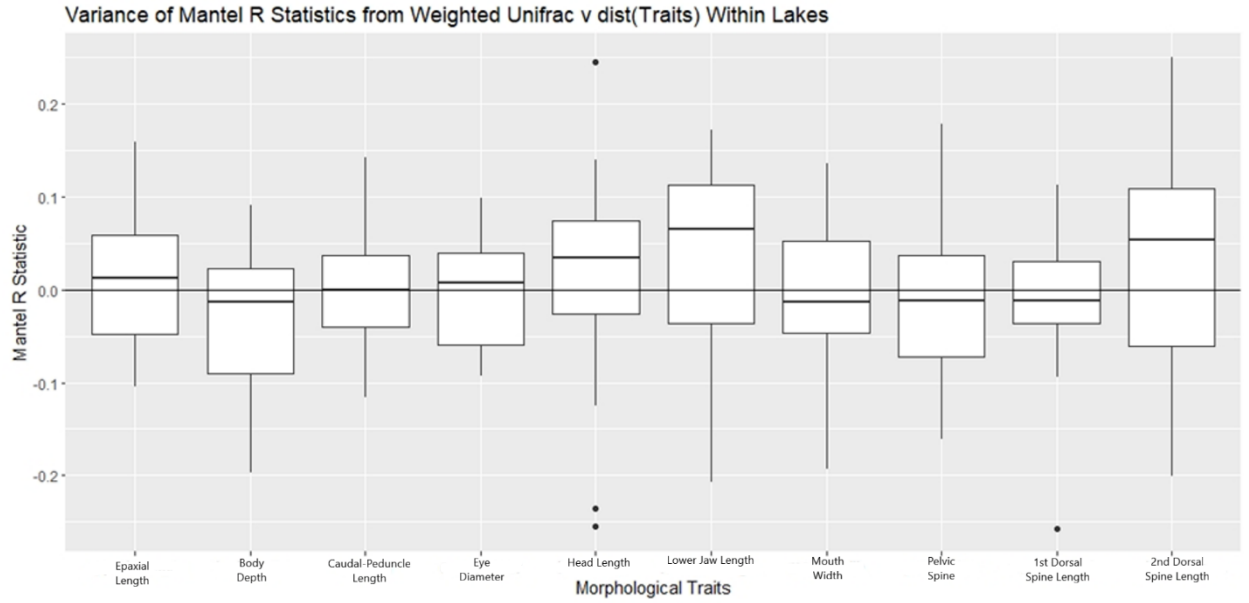

Figure 8.2: Variation in Mantel R statistics generated between each trait's distance matrix and weighted Unifrac. Horizontal line shows the point of no correlation, 0.

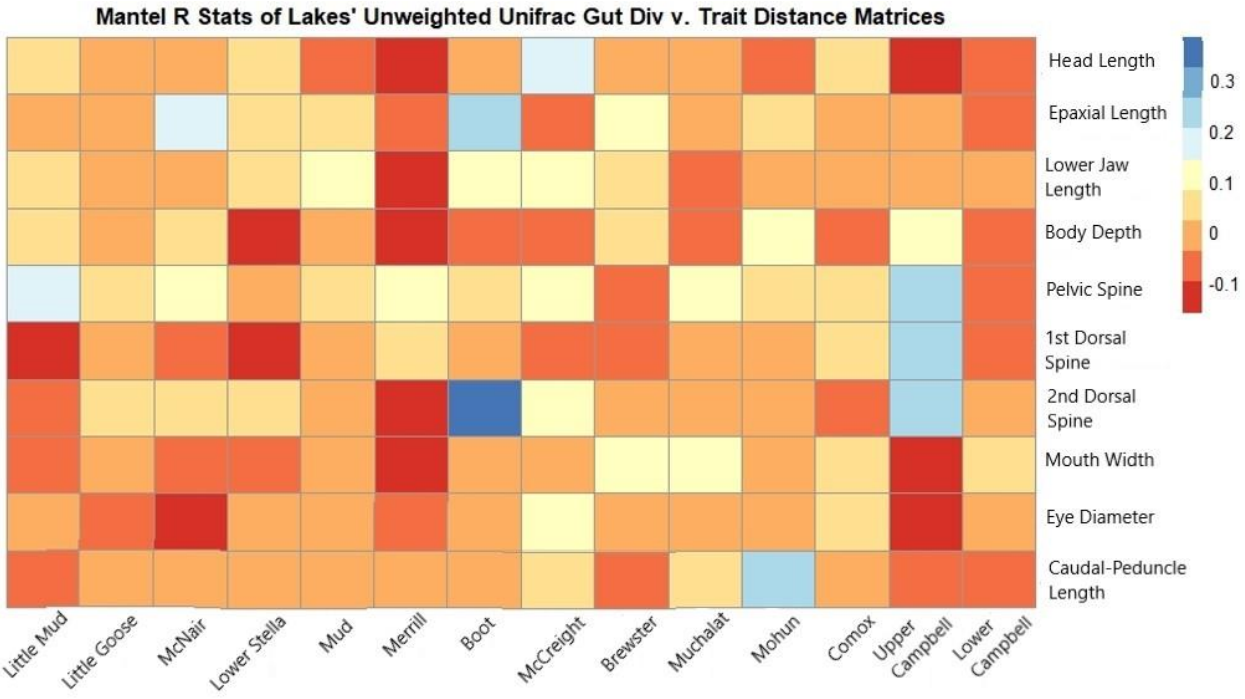

Figure 9.1: Heatmap showing the strength of Mantel R statistics generated between a distance matrix of each trait on the x axis and unweighted Unifrac within each lake. Lakes are in order of increasing surface area on the y axis.

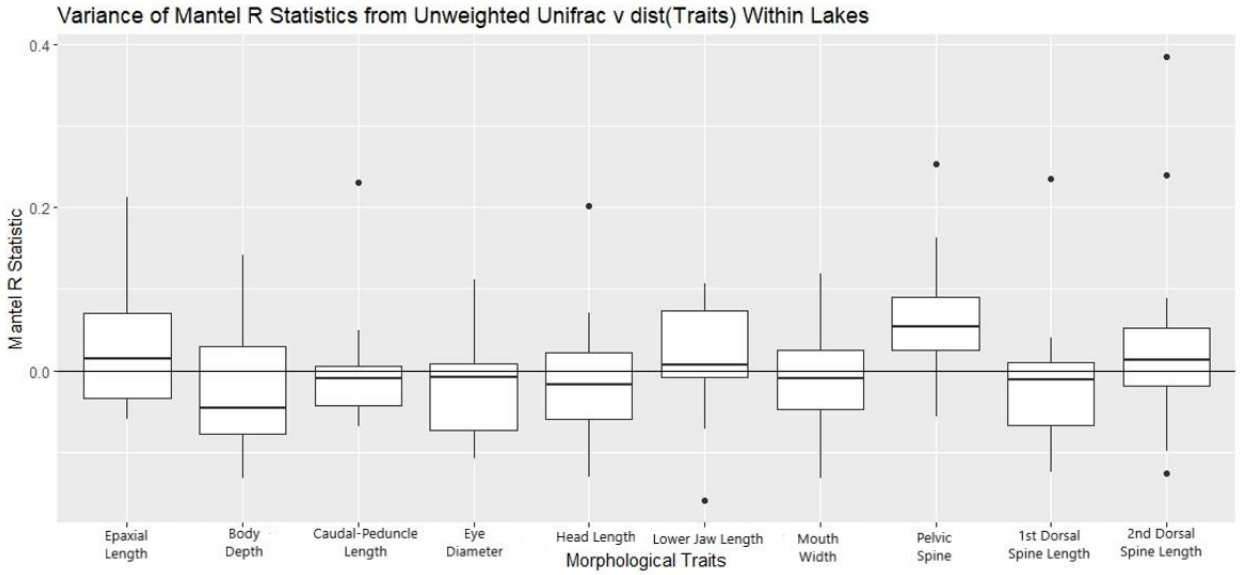

Figure 9.2: Variation in Mantel R statistics generated between each trait's distance matrix and unweighted Unifrac. Horizontal line shows the point of no correlation, 0.

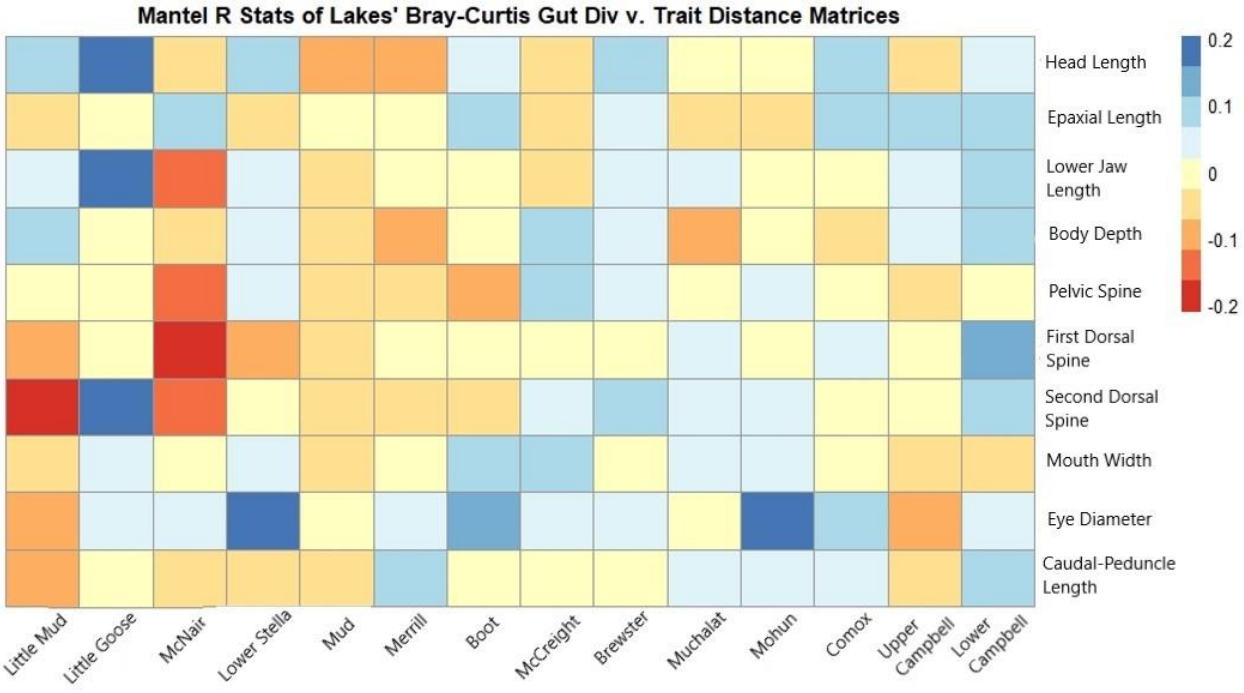

Figure 10.1: Heatmap showing the strength of Mantel R statistics generated between a distance matrix of each trait on the x axis and Bray-Curtis dissimilarity within each lake. Lakes are in order of increasing surface area on the y axis.

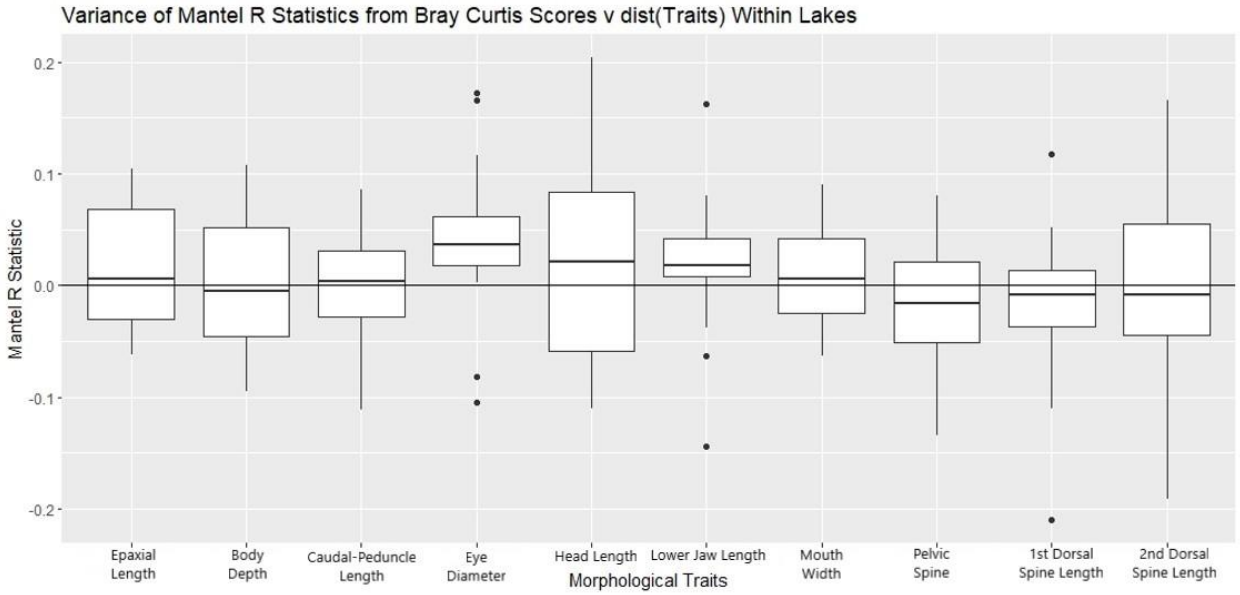

Figure 10.2: Variation in Mantel R statistics generated between each trait's distance matrix and Bray-Curtis dissimilarity. The horizon line shows the point of no correlation, 0.

DISCUSSION

Summary of Results

The goal of this study was to analyze the relationships between trophic ecology (using lake size as proxy), morphological traits, and gut microbiome diversity to identify patterns in the gut microbiome which might reflect niche specialization in threespine stickleback. Bolnick and Ballare (2020) showed that there was a negative linear relationship between mean proportion of benthic prey and lake size- hence our use of it as a trophic proxy. It has also been noted that benthic- and limnetic- feeding fish in sympatric species pairs possess distinct morphological traits (McPhail, 1984; Bell & Foster, 1995) and gut microbiome composition from one another (Rennison et al. 2019). Particular traits have also been shown to correlate with trophic niche. For example, eye diameter is proportionally larger in limnetic fish which is suggested to improve their ability to find small prey in open water (Bell & Foster 1995; McPhail 1984). In accordance with Bolnick and colleagues, limnetic trophic traits may occur more frequently in larger lakes due to the higher ratio of limnetic habitat (2020). Based on this prior work, we hypothesized that morphological traits, particularly those involved in prey acquisition and feeding (e.g., eye diameter, mouth width, head length) may correlate with gut microbiome diversity measures, either across or within populations from lakes of different sizes.

To test for the relationship between morphology and gut microbiome composition we utilized several gut microbiome diversity measures including alpha diversity, which accounts for the number and relative presence of individual bacterial lineages, and beta diversity, which quantifies relative differences in gut microbiome composition between hosts and populations. Firstly, we did not find a suitable linear or quadratic relationship between either the average number of ASVs or the Shannon entropy scores and lake size (Figure 4.1 &4.2, Table 3). Further, few significant correlation coefficients were found between feeding traits and alpha

diversity within lake sizes (Figure 5.1 - 6.2). Nor was a pattern of within lake correlations found across different sized lakes (Figures 5.1 & 6.1). A pattern of correlation coefficients across lake sizes was also generally absent between alpha diversity and morphological PC scores, though within different sized lakes, there were weak correlations between the morphological PC scores and alpha diversity (Figures 7.1 & 7.2). Some beta diversity measures were found to have weakly significant Mantel test R statistics when compared to distance matrices of morphological measurements within lakes, but for all beta diversity metrics there was no discernable pattern for lake size (Figures 8.1 – 10.2). Nor were there any significant relationships between populations' morphological traits and beta diversity (Table 4). These results do not support our hypothesis of a significant relationship between gut microbiome diversity and morphology along the benthic-limnetic axis either within or across lakes of increasing size.

In agreeance with our presumption of the relationship between trophic ecology and morphology, we did find a significant quadratic relationship between the lower jaw length and lateral plate count traits and lake size (Table 2). However, our supposition that variance in morphological traits would peak in intermediate size lakes due to the relatively equal proportion of benthic and limnetic habitat, resulting in a quadratic fit of the data, was not supported (Table 3). The principal component analysis that we conducted of individuals' morphological traits, based on their lake of origin, did identify clustering along PC2 related to watersheds of origin rather than lake size (Figure 3.1). We had hypothesized that populations from lakes of intermediate size, showing the strongest diet divergence between individuals (Bolnick & Ballare 2020), would have the highest gut microbiome alpha diversity. However, this was not found to be the case, with considerable variation in alpha diversity found across lakes regardless of size, and neither linear nor quadratic models found to fit the data (Figures 4.1 & 4.2, Table 4).

Relationship between trophic ecology proxy and morphology

We anticipated a relationship between morphological feeding trait values and trophic ecology using a proxy of lake size. This prediction was based in part on Bolnick and Ballare's 2020 study, which showed a negative linear relationship between proportion of benthic diet and lake size. Yet, no such relationship was found in our data for any of the morphological traits measured. That study also found that the morphological traits measured (mouth width, gill raker size, and gill raker number) were weak predictors of diet (Bolnick & Ballare 2020). This pattern agrees with how nine of the eleven morphological traits we measured did not show a relationship with lake size. We also predicted patterns in morphological variation across lakes along the benthic-limnetic axis due to the established morphological differences between sympatric benthic- limnetic pairs. This is because multiple studies have described morphological differences between sympatric benthic and limnetic fish, which are primarily differ in trophic features: benthic ecotypes are broader and possess wider mouths, while limnetic ecotypes are slimmer, with larger eyes proportional to their bodies (Bell & Foster 1995; McPhail 1984). However, this differentiation may be exaggerated in sympatric species pairs due to character displacement, a mechanism by which resource competition between species is minimized by trait divergence, driving morphological differentiation between competitors (Pfennig & Pfennig 2012; Schluter & McPhail 1992). This effect has previously been observed in stickleback, as a study by Schluter and McPhail (1992) found that four of the six criteria for character displacement were met by the stickleback sympatric species pairs. In fact, the sympatric species pairs were found to be more divergent than isolated allopatric populations in four of five measured traits (Schluter & McPhail 1992). As none of the lakes we surveyed contain sympatric pairs, it is possible that the

absence of character displacement resulted in less pronounced phenotypic differences that did not vary enough across lake sizes to produce a detectable signal. In contrast, Lavin and McPhail (1985) found a relationship between lake size and morphology that was reflective of diet differences amongst the allopatric populations of the Cowichan watershed on Vancouver Island, having measured the standard length, head length, snout length, eye diameter, upper jaw length, gill raker number, gill raker length, head depth, inner orbital width, and plate number. Again, the majority of these traits were not accounted for in our data, but the quadratic relationship we found between lower jaw length and lake size does agree with the results of this study.

The quadratic relationship identified between lateral plates is also of interest, as multiple studies have found that a reduced number of plates correlates with the absence of piscivorous predators (Lavin & McPhail 1985; Moodie 1972; Reimchen 1983). Data on the other members of the trophic web in the lakes sampled is required to test whether the presence of predators affects lateral plate numbers in our study system. We also found via a PCA analysis that watershed was a better predictor of clustering along PC2 than lake size. This pattern requires more investigation, as Lavin and McPhail's 1985 paper detected morphological differences in lakes of different sizes within the same watershed. Absence of a signal between morphology and trophic ecology proxy could also be a consequence of the highly variable trait values observed within each lake, such that the range is nearly the same across lakes regardless of size. As a consequence, there is no pattern to be found within our data set.

Relationship between trophic ecology proxy and gut microbiome

We hypothesized that there may be a relationship between our trophic ecology proxy of lake size and gut microbiome diversity, in part based on the Bolnick & Ballare (2020) study that found a peak in mean pairwise diet difference in intermediate sized lakes- thus, we postulated

that there may be a corresponding peak in gut microbiome diversity to account for the wider assortment of diet items. This supposition was also based on findings from Rennison et al. (2019) wherein it was discovered that sympatric pairs differed in bacterial composition and exhibited consistent differences between sympatric benthic and limnetic stickleback in the inferred functional bacterial gut microbiome. Perhaps here as well the lack of character displacement is in effect- the specialization of gut bacteria of each member of these sympatric species pairs could be enhanced by the presence of the other ecotype (Pfennig & Pfennig 2012; Schluter & McPhail, 1992). Rennison and colleagues (2019) did find in the same study that there were no differences in bacterial species richness across ecotypes, which does agree with our results. As a next step, it would be interesting to test whether gut microbiome divergence is indeed higher in the sympatric species pairs compared to benthic-like and limnetic-like allopatric populations from small and large lakes, as well as looking into the inferred functions of the microbial taxa.

A complication that may have arisen alongside the previously stated mercurial nature of the gut microbiome is the potential influence of climate change, leading to temporal variation in abiotic conditions. Though published in 2020, the samples Bolnick and Ballare used for the diet analysis upon which we predicated our hypothesis, were collected in 2009. In contrast, our samples were collected in the spring of 2020 and 2021. A regional report published by the Canadian government on British Columbia noted that the region as a whole- including Vancouver Island- has experienced an increase in temperature of 1.6°C between 1948 and 2016 across seasons that is continuing to climb; this means fewer days under 0°C and subsequent disruption of established ecological rhythms (Gifford et al. 2022). This abiotic shift may affect trophic interactions, and complicate detection of a link between trophic ecology; particularly

when the analysis is based on diet estimates from samples collected almost 14 years ago and the gut microbiome diversity estimated from samples collected 2-3 years ago. One possible avenue of trophic disruption can be seen in a 2004 study, which showed that increasingly warmer springs since 1962 have disrupted the trophic linkages between phytoplankton and zooplankton, resulting in large blooms of phytoplankton in Seattle's Lake Washington (Winder & Schindler 2004). An increase in the availability of limnetic prey such as phytoplankton could contribute to the lack of patterned variation we predicted for beta diversity across lake sizes (Bell & Foster 1995). Additionally, temperature has been noted to directly influence gut bacteria. For example, in Eurasian lizards (*Zootoca vivipara*) it was found that a 2–3 °C increase in ambient temperatures was associated with a 34% loss of microbiota diversity, primarily in terms of richness (Bestion et al. 2017). As already stated, threespine stickleback habitats we collected from have been experiencing shifts in temperature, although the precise magnitude of change between 2009 and 2020/2021is unclear. Climate change and the effects of subsequent ecosystem disruption since Bolnick and Ballare's specimen collection upon the trophic ecology and thus niche specialization of stickleback may at least partially explain the absence of the predicted correlation between trophic ecology and gut microbiome diversity.

Relationship between morphology and gut microbiome diversity

We hypothesized that there may be a discernable pattern of gut microbiome diversity that correlates with the benthic-limnetic axis, such that alpha diversity scores vary with host trophic ecology along this axis. Further, we hypothesized morphological divergence to be correlated with gut microbiome divergence (beta diversity) within and across lakes of varying sizes. However, there are multiple factors that can impact the microbiome, which complicates our ability to detect the effect of lake size on gut microbiome variation. Firstly, multiple studies have

pointed out the malleability of microbiome composition over time. One study examining soil microbiomes of multiple plant species' mesocosms found that significant temporal variation in bacterial phyla composition occurred as often as on a month-by-month basis (Hannula et al. 2019). Another study investigating the gut microbiomes of farmed Atlantic cod (*Gadus morhua*) found that regardless of dietary treatment, temporal variation of the gut microbiome occurred such that the alpha and beta diversity decreased significantly in a matter of eight weeks (Keating et al. 2021). Further complications arise due to individual level variation, as has been described for humans. Flores et al. found that an individuals' gut diversity varied over the course of three months, and individuals who initially had more diverse communities experienced reduced shifts in bacterial phyla (2014). Overall, the ease with which microbial communities shift, compared to the stability of heritable morphological traits, may mean that gut microbiome diversity and morphology are not adequate predictors of each other (Bell & Foster 1995). Thus, it may be important to look at changes over stickleback life stages or at the stability of certain bacterial taxa in order to find a relationship with morphology if one indeed exists.

Sexual dimorphism may also complicate detection of a relationship between morphology and gut microbiome diversity. Sexual dimorphism is when males and females of a species exhibit different morphological characteristics (Bolnick & Doebeli 2003; Shine 1989). Sexual dimorphism has been documented in stickleback; for example, a study of stickleback from both Japan and Canada found that wild male stickleback specimens have larger mouths and heads, while females have greater standard length and longer pelvic girdles (Kitano et al. 2007). Leinonen et al. (2011) reported that female stickleback have longer dorsal spines than do males. All of these traits were included in our analysis. Further, Bolnick and colleagues (2014) found that diet had sex specific effects on the gut microbiome of stickleback taken from Cedar Lake on

Vancouver Island, such that diet effects in one sex of the species cannot reliably be applied to the other; this was also found to be replicated in Eurasian perch (*Perca fluviatilis*). Indeed, the combined effect of sexual dimorphism in both morphology and gut microbiome composition might have obscured patterns both within lake populations and across those of varying surface area. Correspondingly, in the future it would be worth analyzing the data from this study by sex to see if sex specific effects have obscured patterns across lakes of varying size.

Future directions

Our study of threespine stickleback did not detect a clear relationship between host morphology or gut microbiome diversity and lake size. In follow up analyses of the dataset it would be worth measuring gill raker number and length, as correlations between these traits and trophic level have been reported in numerous other stickleback studies (Lavin & McPhail 1985, 1986; McPhail 1993; Schluter & McPhail 1992). To address the lack of a direct estimate of diet and use of lake size as a trophic ecology proxy, it would be useful to perform an isotope analysis of sticklebacks' muscle tissue to account for the proportions of benthic and limnetic diets more accurately at an individual host level. Isotope estimates would give a better indication of what the fish has eaten over the last months, and therefore be a better measure of its place in the trophic web. This is highlighted by a previous study on Neotropical cichlid fish which showed that divergence in trophic ecology is associated with gut microbiome divergence (Härer et al. 2019). Metabarcoding of the gut contents is another option for determining trophic ecology and would yield a more accurate prediction of diet, as one could identify the exact taxa that each fish consumed. This type of analysis has been used to examine interactions between trophic specialization and morphological divergence in Mexican lake tetras (Ornelas-Garcia et al. 2018). Noting the presence or absence of piscivorous predators would also potentially improve our

ability to explain pattern in lateral plate variation across lakes, and potentially reveal an interesting pattern within the trophic web that may impact gut microbiome diversity. Further analysis is also required to explore the effects of watershed on morphological variation, as seen in the PCA. A functional analysis of the gut microbiome should be conducted, along with a metabolomic study- in the sympatric benthic-limnetic work, a stronger pattern of differentiation was found for inferred function than composition. (Chen et al. 2019; Rennison et al. 2019). The biochemical output of the gut microbiome is the true point of interface between itself and the host, and a closer examination may reveal more ecologically relevant results. Independent statical analysis of each sex could also be implemented in order to remove another confounding factor from the analysis. Overall, it is clear that accounting for all potential factors that may influence patterns of niche evolution is incredibly complex- but such is the nature of studying the natural world.

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