MICROBIAL ASSEMBLAGE DYNAMICS WITHIN THE AMERICAN ALLIGATOR NESTING ECOSYSTEM: A COMPARATIVE APPROACH ACROSS ECOLOGICAL SCALES

by

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ABSTRACT

Understanding the ecological processes that shape species assemblage patterns is central to community ecology. The effects of ecological processes on assemblage patterns are scale-dependent. Research that addresses community patterns at different focal scales may discern distinct conclusions about ecological processes that structure communities. Ideally, studies conducted across focal scales may reflect relevant process-shaping assemblage patterns. Here, I use metabarcoding and shotgun sequencing to determine bacterial assemblage patterns among defined focal scales (micro-, meso-, macro-) within the American alligator (*Alligator mississippiensis*) nesting microbiome. I correlate bacterial assemblage patterns among eight defined compartments within and proximal to alligator nests (microscale), among 27 nests (mesoscale), and across three geographic sampling sites (macroscale), to discern among four main ecological processes (i.e. drift, speciation, selection/filtering, dispersal) that drive bacterial pattern-process dynamics. I hypothesized variation in taxonomic diversity but functional redundancy among defined compartments within alligator nests and between geographic sites. Among all focal scales, bacterial richness (α-diversity) did not statistically differ. In contrast, bacterial composition was unique (β-diversity), with whole nests predicting the largest degree of assemblage variation. Interestingly, functional pathways were redundant within nests. Considering these observed scale-based patterns, alligator nest bacterial assemblages are likely sourced from site-specific reservoirs whose dispersal limitations drive taxonomic differences but are under redundant selection. Critically, the alligator eggshell microbiome is comparably distinct from within-nest assemblages. Bacteria that are found only on the alligator eggshell, are not likely sourced from surrounding environmental

reservoirs and are possibly transferred from the mother's cloaca. I speculate that bacteria found only on the alligator eggshell, with the functional potential to degrade the eggshell surface, are an integrated part of hatching ecology. These findings advance pattern-process dynamics within the field of microbial community ecology and describe processes influencing the American alligator nest microbiome.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I: INTRODUCTION

Disentangling the ecological processes that shape species assemblage patterns is central to community ecology (Vellend 2010; Sydenham et al. 2017). Macarthur (1969) proposed that assemblages of communities were structured by selective pressures. More recent work has proposed a plethora of assemblage-structuring processes, that may be grouped into four processes: ecological drift, dispersal, selection/environmental filtering, and speciation (Chesson 1986; Yodzis 1986; Vellend 2010; Sydenham et al. 2017; Ron et al. 2018). Ecological drift is defined as the changes between communities due to stochastic events, dispersal is defined as the movement of individuals or genes between assemblages, and speciation is the result of mutations becoming fixed within populations over time (Bowler and Benton 2005; Schluter and Conte 2009; Gilbert and Levine 2017; Renault et al. 2018). Selection, more appropriately described as environmental filtering, are the abiotic factors that prevent species from establishing or persisting within a given area (Kraft et al. 2015). The diagnoses of community assemblage pattern-process dynamics are scale-dependent. For example, the scale at which assemblages are studied may lead to distinct scale-specific patterns and, potentially, to spurious process-driven conclusions (Wiens 1989; Kotta et al. 2008; Belmaker and Jetz 2013).

Species assemblage patterns are inherently scale-dependent (Wiens 1989; Terlizzi et al. 2007). The focal scale of a study may be referred to as ‘extent’ and ‘grain’, where the extent is the overall area encompassed by a study, and grain as the study-unit(s) of observation (Wiens 1989). Broad-extent studies may generalize fine grain assemblage patterns. Alternatively, fine-extent patterns may fail to capture broad-extent pattern-

structuring processes (Wiens 1989). Ideally, a study conducted across distinct focal extents may describe the primary system processes (i.e. dispersal, selection, drift, speciation) structuring ecological assemblage patterns (Wiens 1989). These dynamics are not restricted to a particular grain and occur at macro- and microscopic scales (Boon et al. 2013; Nemergut et al. 2013).

Microorganisms are subject to similar, scale-dependent, macro-organismal ecological processes (Boon et al. 2013; Nemergut et al. 2013; Morrison-Whittle and Goddard 2015; Shapiro and Polz 2015). Distinct microbial assemblages may occur within close spatial proximity due to environmental variations at the microscopic scale (Yu et al. 2009; Baxter et al. 2012; Branco et al. 2013; Nemergut et al. 2013; Canfora et al. 2014; Gourmelon et al. 2016). As a result, an individual host, may harbor an abundance of unique microbial assemblages (Caporaso et al. 2011; Colston and Jackson 2016; Gilbert et al. 2018). Together, the total sum of host-associated microbial assemblages and gene products (the metagenome) is defined as the microbiome (Turnbaugh et al. 2007).

Considerations of the microbiome can be approached from both a taxonomic and/or functional perspective (Turnbaugh et al. 2007; Burke et al. 2011; Louca et al. 2016; Louca et al. 2018). For example, distinct taxonomic assemblages may be functionally redundant as similar metabolic pathways are not necessarily clade specific (Burke et al. 2011; Bletz et al. 2016; Louca et al. 2016). Understanding microbial dynamics amongst taxonomic and functional assemblages, across extents, may further discern process-driven assemblage patterns within microbial community ecology (Nemergut et al. 2013; Sydenham et al. 2017; Louca et al. 2018). Namely, assemblages

which are taxonomically distinct yet functionally redundant may be dispersal-limited but subject to similar environmental filters, i.e. selective processes (Jauker et al. 2009; Peay et al. 2010; Burke et al. 2011; Louca et al. 2016; Welsh et al. 2016). Patterns of diversity among assemblages may also be a result of variation in niche availability, competition, and/or colonization legacies within or across extents (Soininen and Heino 2007; Eldridge et al. 2017; Galaiduk et al. 2017). Consequently, community assemblage patterns may represent distinct scale-dependent processes (Levin 1995; Kotta et al. 2008; Lauber et al. 2009; Branco et al. 2013).

Increasing evidence has correlated the microbiome to host ecology (Harris et al. 2006; Sharon et al. 2011; Jacob et al. 2015; Gehring et al. 2017; Kearns et al. 2017; Wiley et al. 2017; Kwak et al. 2018; Flechas et al. 2019). For example, during American alligator (*Alligator mississippiensis*) egg incubation, extrinsic eggshell degradation manifests as an opaque band which migrates across the eggshell surface (Ferguson 1981; Ferguson 1982). Ferguson (1981) postulated that extrinsic degradation observed on alligator eggshells, necessary for embryonic development and hatching success (Ferguson 1982; Joanen and McNease 1989), was caused by nest or eggshell-associated bacterial metabolites (i.e. nest microbiome). If bacteria inhabiting the nest microbiome have the potential to degrade the eggshell surface they may positively influence not only alligator hatching success but, an assortment of species including wading birds, small mammals, and other reptiles, indirectly due to predation (Craighead 1968; Joanen 1969; Palmer and Mazzotti 2004; Merchant et al. 2014).

Here, I characterize bacterial taxonomic assemblages and functional pathways, across extents (Fig. 1), to infer the ecological processes structuring the American alligator nest microbiome. Three seemingly distinct extents occur within the alligator nesting environment. The micro-extent specifies bacterial assemblages among delineated compartments within and proximal to individual nests (Fig. 2). The meso-extent is a homogenization of bacterial assemblages among nests, while the macro-extent are whole assemblages among distinct geographic sites (Fig. 3). My objectives were to: 1) characterize bacterial taxonomic assemblages and functional pathways across extents of the alligator nesting microbiome; 2) infer the ecological processes, that structure bacterial taxonomic assemblages and functional pathways of alligator nests and; 3) propose environmental reservoirs which contribute to nest and eggshell assemblages. I hypothesized that: 1) alligator nests would host distinct taxonomic bacterial assemblages yet be functionally redundant across extents; 2) taxonomic assemblages would be dispersal-limited and maintained due to environmental filtering but redundant selective processes would structure nest functional pathways, and; 3) nest and eggshell assemblages would be colonized by site-specific bacteria. This research advances our understanding of bacterial community ecology and describes factors influencing the American alligator nesting microbiome.

CHAPTER II: METHODS

Study Species – The American alligator (*Alligator mississippiensis*) is a charismatic reptile found throughout the coastal southeastern United States (Goodwin and Marion 1979; Ducarme et al. 2013). American alligators are keystone species serving as apex predators and ecosystem engineers (Craighead 1968; Kushlan 1974; Mazzotti et al. 2009). Alligators typically lay their eggs in June and early July, although mean oviposition date can significantly vary among years (Kushlan and Jacobsen 1990). Eggs are typically deposited into mounds constructed of nearby vegetation (Joanen and McNease 1989). Female alligators often remain in attendance and may guard their nests throughout incubation (Kushlan and Kushlan 1980b) and will periodically mount, compact, and excrete waste onto the nest on occasion (LeBuff Jr 1957; Joanen 1969). Finally, females may assist hatchlings as they emerge from the egg, transport them to a nearby water source, and act as a predator deterrent (Kushlan 1973). Alligator nests are also frequently used by numerous other species in an array of associated behaviors (Kushlan and Kushlan 1980a; Merchant et al. 2014).

During egg development, an initial small opaque band appears within 24 hours of deposition on the top medial surface of fertile eggs that is thought to occur by extrinsic degradation from bacterial metabolites within the nesting environment (Ferguson 1981; Ferguson 1982). After four days, an opaque band appears that extends around the top half of the egg. A second, fusiform, opaque band forms around the egg at day 30 and completely covers the egg surface after day 45; eggs on average hatch at day 65

(Ferguson 1982). Removal of alligator eggs from the nest media may reduce hatching success (Joanen and McNease 1989).

Sample Collection **–** Twenty-three nests were sampled at J.D. Murphree Wildlife Management Area (J.D. WMA, 8 in 2017 and 15 in 2018). Two nests were sampled at Eufaula National Wildlife Refuge (ENWR) and three nests were sampled within the Mobile-Tensaw Delta (MTD, district 5) in 2018 (Fig. 3). Individual alligator nests were sampled at five defined compartments (A-C, E-F; Fig. 2) within nests, surrounding water (D; Fig. 2), and a single nest-specific egg (G; Fig. 2). For the 2018 field season, an additional sample (H) was collected along the nest dripline (Fig. 2). Nests without eggs were sampled at compartments A and B exclusively (two nests in 2017 and five nests in 2018). Sampled nests at ENWR were atypically constructed of mud with little to no vegetation cover and therefore prevented the collection of sample B from these nests (Grajal-Puche et al. 2018).

Sample Collection for high-throughput sequencing **–** All samples (A-H, Fig. 2) were collected with sterile polyester tipped swabs (Puritan©, SKU#: 25-806 1PD SOLID). Swabs were inserted into the defined nest compartments (A-C, E-F; Fig. 2) or proximal environments (D, H; Fig. 2) and rotated for 15s. Eggshell surfaces (G) were rinsed for 15s to remove large transient debris using sterile Millipore water autoclaved for two hours. Eggshell samples were collected using 15 swab strokes by rotating a sterile swab across a five cm area of each egg. Within-nest and eggshell samples (A-C, E-G; Fig. 2) were collected in a stratified fashion, commencing at the nest exterior (A; Fig. 2) and progressively sampling deeper (B-C, E-G; Fig. 2). Nest-associated water and dripline

samples (D, H; Fig. 2) were collected after all other samples had been stored at 4º C. All samples (A-H; Fig. 2) were stored at -20ºC until processing.

DNA extraction, High-throughput Sequencing and Bioinformatics – DNA was extracted from 170 swab samples using a Qiagen PowerSoil HTP96 kit according to the manufacturer's instructions. A single 'environmental extraction blank' was collected in the field by exposing a swab to the air, gloves and eggshell rinse water and included on each of the two 96 well plates to control for contamination. DNA extraction and library preparation was completed in separate AirCleanSystems AC600 (AirClean Systems, Creedmoor, NC) dedicated to each process. Each workstation had a dedicated set of pipettes that were routinely autoclaved and UV crosslinked prior to DNA extraction and sequencing.

Two library preparations were completed including 16S amplicon sequencing of the bacterial rRNA gene to characterize taxonomic microbial assemblages and shotgun metagenomic sequencing to characterize functional gene pathways. For amplicon sequencing, the Illumina 16S Metagenomic Sequencing Library Preparation protocol was used and libraries were sequenced on the Illumina MiSeq platform in three separate sequencing runs. The V4 region of the 16s rRNA gene was amplified as in Kozich *et al.* (2013). Ampure XP magnetic beads were used to clean DNA samples both at initial amplicon and index PCR steps. Post-amplification, samples were quantified using the Promega QuantiFlour ONE dsDNA System per the manufacturer's protocol (Truman and Wieczorek 2013). After quality control and normalization of DNA, samples were sequenced using the Illumina MiSeq v2 flow cell and sequenced on a 500-cycle reagent

kit (PE 2X250). A total of 25 257 358 raw data sequence reads were obtained from all three runs. Bioinformatics processing was conducted according to Kozich *et al.* (2013) on the *mothur* platform v.1.41.2 (Schloss et al. 2009). Contigs with less than 253 bp or greater than 254 bp, containing eight or more homopolymers, and any ambiguities were discarded from the analysis. The remaining sequences were aligned to the SILVA v. 132 bacterial reference database (Quast et al. 2013). Chimeras were removed with VSEARCH (Rognes et al. 2016) and any sequences identified as Archaea, Eukaryota, chloroplast, mitochondria, or unknown were discarded from the data set. The *opti* method was used in *cluster.split* to cluster operational taxonomic units (OTUs) at 97% sequence similarity. Rare OTUs appearing fewer than 50 times and OTUs from 'environmental contamination' extraction blanks were removed. A total of 9 648 888 sequences were retained after filtering and quality control. The resulting data set was subsampled at 3 241 sequence reads per sample to normalize sample coverage. Shotgun library preparation was conducted according to the Illumina Nextera DNA Flex Library Preparation reference guide for 17 nests including 34 samples corresponding to the nest exterior (A; Fig. 2; n = 17) and egg-chamber after ~50% of the eggs were removed (E; Fig. 2; n = 17). Isolated DNA was tagmented using a bead linked transposome process, cleaned up using Ampure XP magnetic beads, PCR amplified to add i7and i5 sequencing indices, normalized, pooled and sequenced on a NextSeq 500 using a High Output v2.5 (150 cycles; 2x75 paired-end reads) kit. A total of 7.53 Giga base pairs were obtained from the run. During bioinformatics processing, I concatenated forward and reverse paired-end reads together using *concat_paired_end.pl* in the Microbiome Helper package (Comeau et al. 2017). I used *Trimmomatic* with a sliding window of 20 bp and minimum length of

50 bp to determine reads to be kept. *Bowtie2* was run using the parameters *very-sensitive* and *dovetail* to remove human and/or PhiX contaminating reads. *MetaPhlAn2* (Segata et al. 2012) was used to assign taxonomy to reads and *HUMAnN2* (Franzosa et al. 2018) to categorize functional pathway using the *UniRef50* and *MetaCyc* reference databases. After assigning functional gene pathways to each sample, the complete data set was normalized into relative abundance data for statistical analysis in R (see below).

CHAPTER III: STATISTICAL METHODS

Microbial assemblage patterns within the alligator nest microbiome were analyzed among micro- (samples within or proximal to the nest environment, A-H; Fig. 2), meso- (among-nest bacterial assemblages), and macro-extent (among-site bacterial assemblages) factors. All statistical analyses were conducted in R version 3.5.1 (R Core Team 2019) using packages '*vegan'*, which provides descriptive tools for community ecology (Oksanen et al. 2019), '*rcompanion'*, which produces a table of statistics for multiple statistical models (Mangiafico 2019), '*ggplot2'*, a package used to create visual data plots (Wickman 2016), and '*indicspecies'*, that provides functions to assess the strength and significance between species abundance/occurrence patterns and groups of sites (De Cáceres and Legendre 2009). Bacterial taxonomic patterns and functional pathways were compared among defined extents to understand the effect of scale-based processes on assemblage patterns within the alligator nesting microbiome.

***Taxonomic patterns* –** Using a Kruskal-Wallis or ANOVA, I compared the calculated inverse-Simpson's diversity metrics (α-diversity) from OTU assemblage sequence reads within alligator nests (micro-extent, Fig. 2), among nests (meso-extent), and among geographic sites (macro-extent). ANOVA was used to compare whether group-specific samples were differentially diverse among extents (micro-, meso-, macro-). For example, assemblage diversity metrics sampled from the exterior surface of an alligator nest (A; Fig. 2) were compared to eggshell assemblages (G; Fig. 2). Prior to statistical evaluation, diversity metrics were tested for normality and appropriately evaluated either utilizing a Scheirer-Ray-Hare test or a two factorial ANOVA design. Bacterial composition (β –

diversity) was compared across extents (micro-, meso-, macro-) by generating a Bray-Curtis dissimilarity matrix from bacterial sequence abundance reads and statistically analyzed using a permutational multivariate analysis of variance (PERMANOVA). Due to differences between whole alligator nests and environmental assemblages, I determined diversity and compositional patterns across all extents (micro-, meso-, macro-; Fig.1) and within-nest (A-C, E-G; Fig.2) assemblages after excluding assemblages from environmental samples (D, H; Fig. 2).

Functional patterns **–** Bacterial functional pathways were analyzed within nests and across sites (extents). Within-nest comparisons were made between functional assemblages sampled at the exterior nest surface (A; Fig. 2) and the inner egg chamber when ~50% of the eggs were removed (E; Fig. 2). Samples A and E were selected for functional analysis as they constituted two physically separated locations with potentially distinct taxonomic assemblages and functional pathways that could represent the summation of whole nest processes. A total of 17 egg-containing nests (n=3 from 2017, n=14 from 2018) were utilized for functional analysis. Functional comparisons among extents (micro-, meso-, macro-) were made by generating Bray-Curtis dissimilarity matrices from functional gene relative abundances and compared using a PERMANOVA test. Functional pathways were visualized with a principal coordinate analysis (PCoA; Fig. 8) generated from dissimilarity matrices.

CHAPTER IV: RESULTS

Taxonomic patterns – Bacterial α-diversity (richness) did not differ between nests and environmental samples (X^2= 8.70, df = 7, p > 0.05), among all nests (X^2= 26.19, df = 26, p > 0.05), or among all geographic sampling locations (X^2 = 1.51, df = 2, p > 0.05). I chose to re-analyze samples exclusively collected within-nests (micro-extent), due to differences in composition between nest and environmental assemblages ($F_{2, 129}$ = 3.503, R^2 = 0.052, p < 0.05; Fig. 4). The removal of environmental samples did not alter α-diversity patterns within-nests ($F_{5, 120}$ = 1.79, p > 0.05). At the mesoscale within J.D. WMA site, bacterial α-diversity among nests did not differ (X^2 = 23.54, df = 21, p > 0.05). Alligator nest bacterial diversity was assessed across extents (micro-within nests, meso-among nests, macro-among geographic sites) to determine if particular patterns were dependent on focal scale. Regardless, α-diversity patterns across extents remained consistent (p > 0.05).

In contrast to species richness (α-diversity) of bacteria, β-diversity significantly differed among alligator nest compartments (micro- $F_{5, 122}$ = 1.809, R^2= 0.072, p < 0.05) among whole nests (meso- $F_{26, 122}$ = 2.357, R^2= 0.390, p < 0.05; Fig. 6) and among geographic sites (macro- $F_{2, 122}$ = 3.672, R^2= 0.058, p < 0.05). Bacterial assemblages within nests were unique relative to assemblages proximal to the nest structure (D, H; Fig. 2) ($F_{2, 129}$= 3.363, R^2 = 0.050, p < 0.05), as were assemblages within-nests from J.D. WMA ($F_{5, 98}$= 1.826, R^2 = 0.089, p < 0.05; Fig. 5). In addition, bacterial assemblages on the eggshell were distinct relative to other within-nest assemblages ($F_{1, 121}$= 2.425, R^2 = 0.020, p < 0.05, Fig. 7). Bacterial composition among nests (meso-) was also unique ($F_{26,}$

$_{104}$= 2.335, R^2 = 0.438, $p < 0.05$) between years across sites ($F_{1, 122}$= 4.168, R^2 = 0.033, $p < 0.05$) and exclusively at J.D. WMA ($F_{1, 98}$ = 4.248, R^2 = 0.042, $p < 0.05$). Whole nest (excluding eggshell samples) and eggshell bacterial assemblages varied between years ($F_{1, 84}$ = 3.922, R^2 = 0.037, $p < 0.05$; $F_{1, 17}$ = 1.394, R^2 = 0.080, $p < 0.05$). Whole-site bacterial composition was distinct among sites ($F_{2, 122}$ = 3.672, R^2 = 0.058, $p < 0.005$; Fig. 6). There was no interactive effect of geographic sites on bacterial taxonomic patterns within the alligator nesting system ($F_{9, 122}$ =1.03, $p > 0.05$).

Functional patterns – Functional gene pathways were redundant between the egg chamber and exterior nest surface ($F_{1, 33}$ = 0.916, $p > 0.05$; Fig. 8). Interestingly, geographic site (macro-extent) functional pathways were statistically distinct ($F_{2, 33}$ = 2.59, R^2 = 0.143, $p < 0.05$; Fig. 8). The atypical construction (mud nests) of sampled nests at ENWR warranted comparisons between J.D. WMA and MTD (vegetation nests). With nests from ENWR excluded, functional pathways were redundant between geographic sites ($F_{1, 29}$ = 1.125, $p > 0.05$). Similarly, within-nest functional pathways from J.D. WMA were also redundant ($F_{1, 25}$ = 0.923, $p > 0.05$).

CHAPTER V: DISCUSSION

Community ecologists have long sought to understand the underlying ecological processes structuring observed species assemblage patterns. Study-scale fundamentally impacts observed patterns and thus leads to scale-specific process-driven conclusions (Wiens 1989). Studies spanning multiple focal scales (i.e. extents) may accurately describe pattern-process dynamics within a study system. During this study, I attempted to diagnose which class of processes (dispersal, drift, speciation, selection/filtering) shaped bacterial composition and functional patterns, within the American alligator nesting environment. Overall, taxonomic bacterial richness (α-diversity) remained uniform within and among nests (micro- and meso-extent), and across sites (macro-extent), yet varied in composition (β-diversity) across all extents (micro-, meso-, and macro-extent). Moreover, functional pathways were redundant within-nests (micro-extent) and unique across sites (macro-extent), indicating that perhaps microbial community ecologists should consider the characterization of microbes from both taxonomic and functional perspectives (Gibbons et al. 2017).

Within the alligator nesting microbiome, taxonomic composition was associated with sampling location across extents, for example, community dissimilarity increased with spatial distance (micro-, meso-, macro-). Within-nest bacterial assemblages were distinct from the external environment. These patterns were not surprising, as host-associated assemblages may be unique relative to free-living bacteria (Arùjo et al. 2002; Fitzpatrick and Allison 2014; Loudon et al. 2014). In addition, I observed a significant amount of bacterial assemblage turnover within alligator nests (micro-extent). This

conflicted with what I expected given the documented ease of microbial dispersal across small spatial scales (Finlay and Clarke 1999; Darcy et al. 2011; Wilkinson et al. 2012; Mikaelyan et al. 2017). Taxonomic turnover within alligator nests (micro-extent) may be driven by other scale-relative selective processes or environmental filters, such as priority effects. Priority effects signify the relative difficulty for a species to establish within a given area due to the succession of previous colonizers (Telford et al. 2006; Andam et al. 2016). Consequently, current assemblages may be relicts of colonization history (Fukami et al. 2010; Peralta et al. 2013). Within alligator nests, separate compartments may be initially colonized by a subset of microbes whose arrival legacy, in part, accounts for assemblage patterns (Fukami et al. 2010; Loudon et al 2014; Andam et al. 2016; Svoboda et al. 2018), although this conclusion would require controlled experimental testing.

In contrast to bacterial taxonomic composition, functional pathways based on metabolic gene pathways were similar within alligator nests (micro-extent, Fig. 8). Redundant functional pathways may be subject to similar environmental filters (Louca et al. 2016; Louca et al. 2018). Relative to my study, redundant functional pathways may indicate that taxonomically diverse bacterial assemblages have similar functional potentials due to analogous selective processes. Therefore, arrival legacy may account for unique taxonomic patterns while horizontal gene transmission may describe within alligator nest (micro-) functional redundancy (Hehemann et al. 2016; Louca et al. 2018). Macro-extent functional pathways contrasted with those at the micro-extent as they were distinct across geographic sites possibly reflective of female alligator reproductive plasticity.

Reproductive plasticity is the change in an individual's reproductive behaviors and or performance due to environmental stimuli (Huang et al. 2017). Plastic nesting ecology may indirectly shape microbial assemblages through altered thermal gradients possibly caused by nest structural differences (Murray et al. 2013; Murray et al. 2016). Nest structural differences may select for functionally distinct bacterial pathways (Maigetter and Pfister 1975; Kamel 2013; Murray et al. 2016). Structural differences among crocodilian nests is relatively common and was observed during the 2018 field season (Murray et al. 2013; Grajal-Puche et al. 2018; Merchant et al. 2018), which may support our conclusions about distinct taxonomic patterns and functional pathways among geographic sites. The removal of functional pathways sampled from atypical nests at ENWR resulted in functional redundancy among geographic sites (macro-extent). Therefore, reproductive plasticity in physically distinct nests (ENWR) may result in dissimilar taxa with unique functional pathways (Kamel 2013; Gourmelon et al. 2016; Lin et al. 2016; Akresh et al. 2017).

Microbial habitats have been determined to be scale-relative enabling distinct environments or filters, to occur within close spatial proximity (Fierer and Lennon 2011; Nemergut et al. 2013; Lear et al. 2014; Gourmelon et al. 2016). Crocodilian nests have distinct compartment-specific thermal patterns (Magnusson 1979; Murray et al. 2016) which may impose strong environmental filters and contribute to taxonomic assemblage turnover (Mouchet et al. 2013; Hu et al. 2019). Stochastic thermal fluctuations, within nest compartments may also promote processes shaping bacterial richness (α-diversity) patterns. For example, crocodilian nests experience a high degree of stochastic events in the form of thermal variations, within and among nests, at the daily and seasonal scale

(Escobedo-Galavan et al. 2016; Lopez-Luna et al. 2015; Murray et al. 2016). Stochastic events may promote colonization of less-dominant microbial taxa, possibly through relaxed competition (Hibbing et al. 2010; Eldridge et al. 2017) and consequently homogenize assemblage richness (α-diversity), which was observed across extents (micro-, meso-, macro-) within the alligator nest system (Belotte et al. 2003; Mikaelyan and Brune 2017; Vega and Gore 2017). Interestingly, among-nest patterns accounted for over a third of the described assemblage variation. This may indicate that mesoscale processes, such as dispersal limitations, primarily underly patterns within the alligator nest system. Patterns of dispersal limitation have been documented in other systems showing that increased distance is positively correlated with assemblage dissimilarity (Sydenham et al. 2017; Peláez and Simone Pavanelli 2018). Distance alone may not entirely account for patterns of bacterial assemblage turnover at the mesoscale and may be attributed to a host's ecology (Conlon and Bird 2015, Ruiz-Castellano 2016).

Alligator ecology may indirectly affect bacterial assemblage patterns among nests. Typically, alligator nests are mounds composed of surrounding vegetation and newly constructed between years (Joanen and McNease 1989). Here, I documented significant assemblage turnover among nests (meso-extent) and years. Alligator nest turnover patterns may indicate that females indirectly shape nest bacterial assemblages by unintentionally drawing from proximate bacterial reservoirs during nest construction (Berg and Smalla 2009; Loudon et al. 2014). During alligator nest construction, initial colonizers may define taxonomic patterns among nests. A meta-analysis of microbial communities revealed that generalist microbes are crucial for initial-site colonization (Sriswasdi et al. 2017), suggesting that alligator nest assemblages are principally

composed of bacterial generalists sourced from the surrounding environment. Generalists also primarily respond to spatial variables (i.e. site locations relative to one another, Pandit et al. 2009) which is consistent with assemblage composition among alligator nests. Critically though, microbial generalist characteristics do not describe eggshell associated assemblages.

Microbial specialists primarily respond to environmental, i.e. selection, rather than spatial, i.e. dispersal, processes (Pandit et al. 2009). I was able to identify a suite of bacteria indicative of the eggshell microbiome which may demonstrate that eggshell-associated bacteria are specialized to this unique calcified environment composing the eggshell surface (Hincke et al. 2012). The relatively high proportion of bacteria specific to the alligator eggshell relative to other within-nest assemblages, supports this observation. Of the 16 OTU's indicative of the eggshell microbiome, *Caulobacteraceae* and the *Alphaprotobacteria* could potentially benefit overall alligator hatching ecology. Both the *Caulobacteraceae* and *Alphaprotobacteria* are chemo-organotrophic, which associate with eukaryotes (Batut et al. 2004) and have the metabolic potential to degrade amino acids on the eggshell surface (Clark and Van Zyl 1976; Schweitzer et al. 2001). Furthermore, I identified 13 indicative functional pathways within the egg chamber (in direct contact with eggshell surface). Of these pathways, two (e.g. Glutaryl-CoA and L-tyrosine degradation) have been documented in substrate degradation. I hypothesize that microorganisms expressing these pathways have the functional potential to degrade the alligator eggshell surface during incubation and aid in hatching success. The distinct possibility exists for transmission of taxa, indicative of the eggshell, from mother alligators to the eggshell during oviposition.

Olsen et al. (2017) suggested the eggshell microbiome reflects the status of the cloacal environment, with sample differences caused by post-lay contamination. Additional evidence suggests that cloacal contact may facilitate assemblage transmission (Kreisinger et al. 2015). Interestingly, there was a relatively high degree of temporal (between years) eggshell assemblage convergence relative to assemblages within-nests. This may indicate that conserved eggshell assemblages are differentially sourced relative to within nest composition. Deductively, bacterial turnover between the eggshell and surrounding nest environment, assemblage convergence across eggshell samples and between years, and the proportion of OTU's that are specific to the eggshell, suggest that a subset of bacteria inhabiting the eggshell surface are not sourced from the surrounding environment but possibly from the maternal cloaca. These insights have ecological implications, as Ferguson (1982) postulated that bacteria on or proximal to the eggshell surface were sources of extrinsic eggshell degradation observed during incubation and were critical for hatching success. The sources of alligator eggshell microbes may also have wildlife management implications.

Bacterial assemblage composition may be shaped by the same extrinsic environmental processes governing macro-ecological systems (Trevelline et al. 2019). Perturbations to the environment, with regards to the alligator nest system, may potentially alter both bacterial taxonomic composition and/or functional pathways (microbiome). Processes structing alligator nest bacterial assemblage patterns is therefore an essential consideration when developing wildlife management practices (Trevelline et al. 2019), as alterations to the alligator nest microbiome has the potential to negatively

impact hatching ecology (e.g. eggshell degradation and hatching success) and alligator populations.

Processes shaping bacterial assemblage patterns within the alligator nesting microbiome are multifaceted and differ both functionally and taxonomically across focal extents. Making observations across space may allow predictions of the primary processes driving assemblage patterns (Wiens 1989; Jin et al. 2019). Here, I analyzed bacterial assemblage patterns across three defined extents within the American alligator nesting microbiome to correlate process-pattern dynamics. Bacterial richness (α-diversity) was uniform yet uniquely composed (β-diversity) across all extents, with individual nests predicting the largest degree of assemblage variation. Interestingly, our analyses did not support the hypothesis of redundant functional pathways among geographic sites; however, functional pathways were redundant within nests. Alligator nest taxonomic composition may be correlated with alligator reproductive plasticity. Female alligators may also shape eggshell assemblages through horizontal transmission and therefore influence hatching success. Future studies should characterize bacterial assemblages between the eggshell and mother cloaca microbiome to specify processes effecting bacterial assemblages within alligator nests. Additionally, identifying the effect of functional dysbiosis among alligator nests on alligator hatching success may have applicable conservation ramifications. Nevertheless, these findings disentangle specific extent-based processes which shape alligator nest bacterial patterns and serves as a foundation for ensuing microbial community ecology research.

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APPENDICES

Appendix A: Figures

Sampling extents

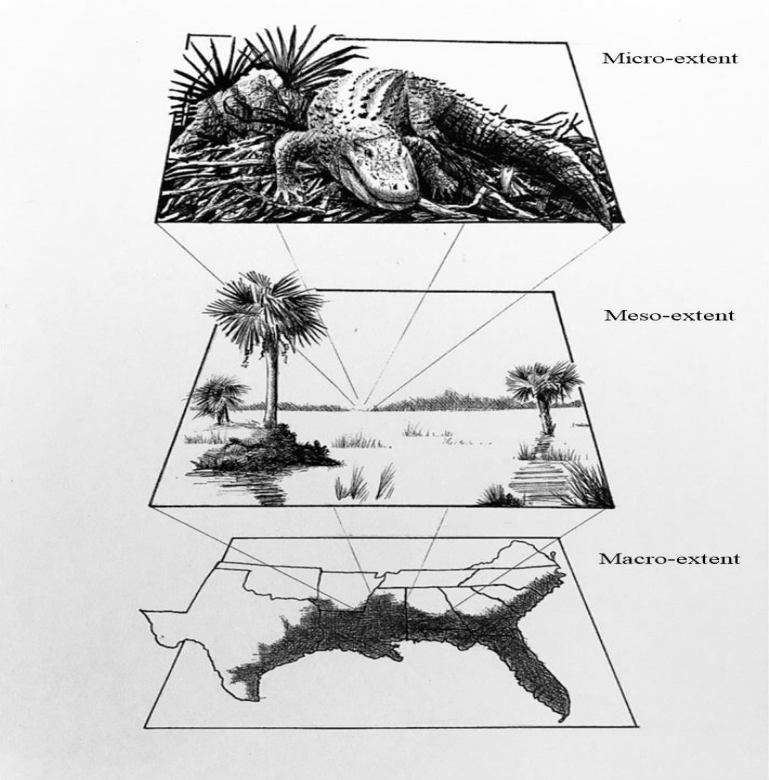

Figure 1: Sampling design across extents (micro-, meso-, macro-). Micro-extent compared bacterial assemblages within nests. Meso-extent bacterial assemblages were compared among nests. Macro-extent compared bacterial assemblages among geographic sites. Grajal, A. 2019a.

Sampling design

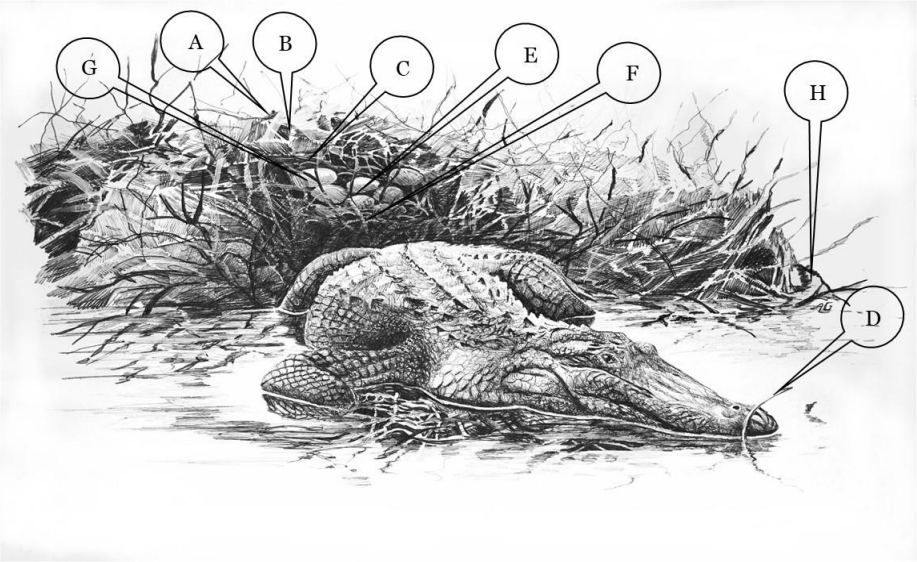

Figure 2: Sampling locations within alligator nests (micro-extent). Letters signify samples collected from: A – nest exterior surface, B – five centimeters beneath exterior surface, C – above egg-chamber, D – surrounding water, E – egg-chamber after ~50% of eggs removed, F – bottom of egg-chamber after ~100% of eggs removed, G – eggshell surface, H – nest dripline. Grajal, A. 2019b.

Geographic sampling locations

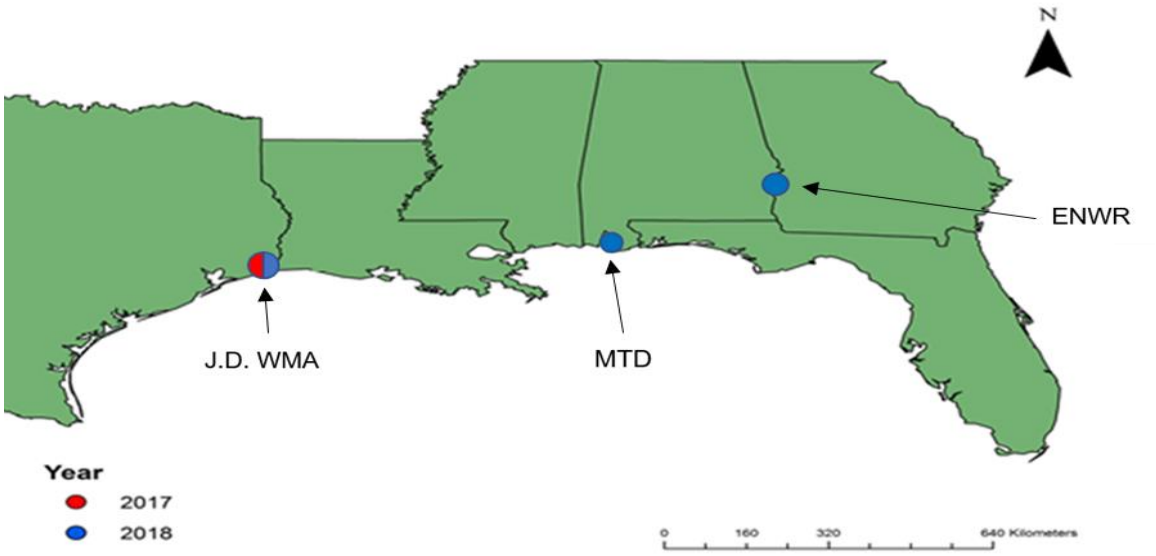

Figure 3: Geographic sampling locations (macro-extent). J.D. Murphree Wildlife Management Area (J.D. WMA), Mobile-Tensaw Delta (MTD), Eufaula National Wildlife Refuge (ENWR). J.D. Murphree WMA was sampled across years.

Bacterial assemblages within nests and the surrounding environment

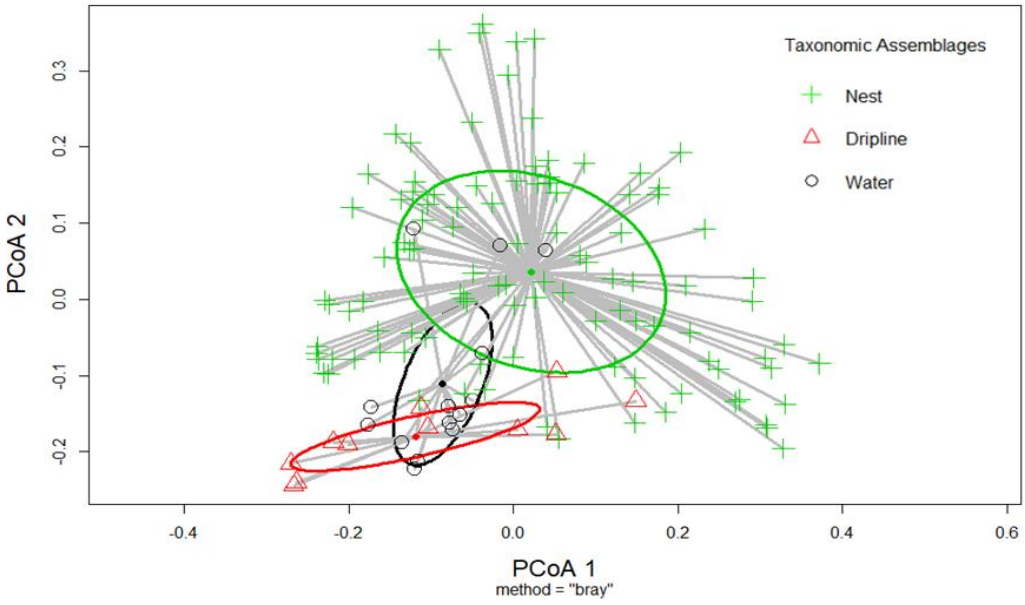

Figure 4: Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity matrices among nest and environmental bacterial assemblages. Points represent bacterial assemblages within each sample-type (i.e. nests, nest dripline, and surrounding water). Ellipses encircle the mean assemblage dispersion. PERMANOVA results indicated that the microbial assemblages were statistically different between sampled habitats ($F_{2,129}$=3.503, R^2 =0.052, $p < 0.05$).

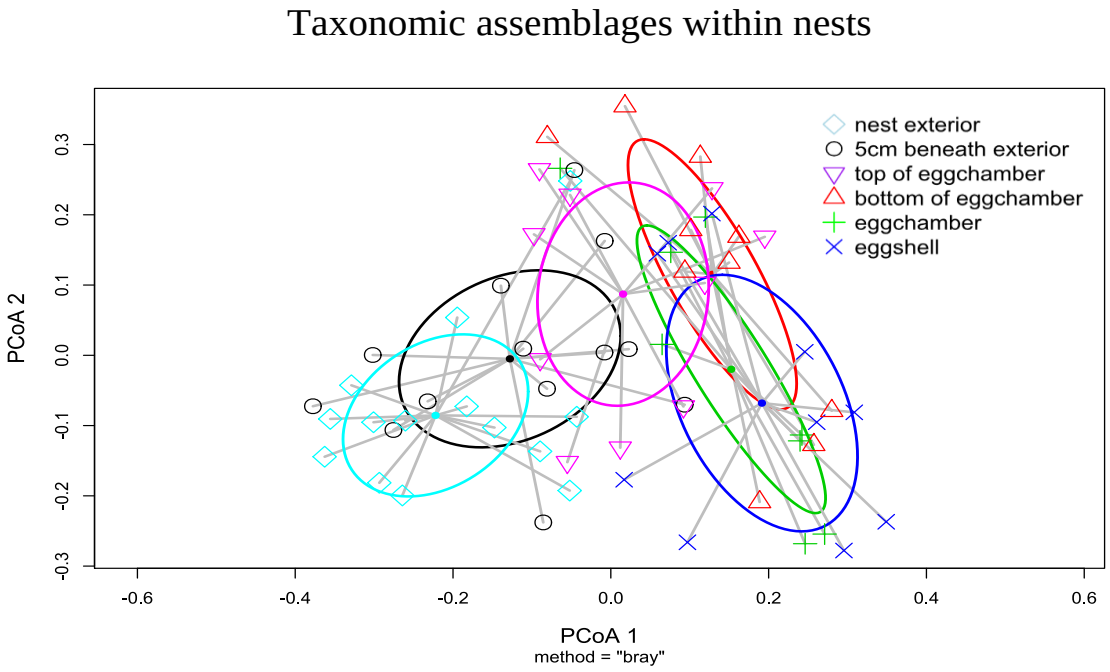

Figure 5: PCoA of bacterial assemblages among nests sampled in 2018 from J.D.WMA. Points represent specific bacterial assemblages within each sample-type (defined in legend). Ellipses encircle the mean sample-type assemblage dispersion. Dud nests (n = 8) and samples collected from the nest dripline (n = 11) and surrounding water (n =14) are not included. PERMANOVA results indicated that the microbial assemblages were statistically different at the microscale ($F_{5,98}$ = 1.826, R^2 = 0.089. p < 0.05).

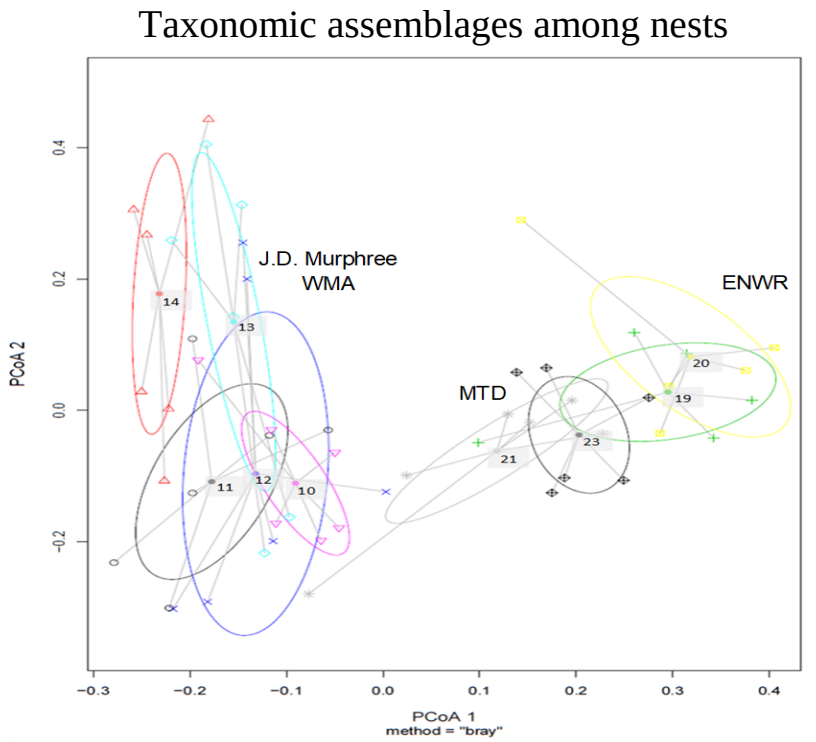

Figure 6: PCoA among nests sampled in 2018. Points represent taxonomic bacterial assemblages within associated nests. Ellipses encircle the mean assemblage dispersion. Dud nests (n = 8) and samples collected from the surrounding nest environment (n = 14) are not included. Only J.D. WMA samples collected on the same day (10-14) were included in this analysis to remove day-to-day temporal variation. Numbers within ellipse centroids correspond to independent nests. Samples 10-14, 19-20, 21, 23 were collected at J.D. WMA, ENWR, and MTD, respectively. PERMANOVA indicated that microbial assemblages were statistically different between geographic locations at the macroscale ($F_{8,42}$ = 2.316, R^2 = 0.353. p < 0.05).

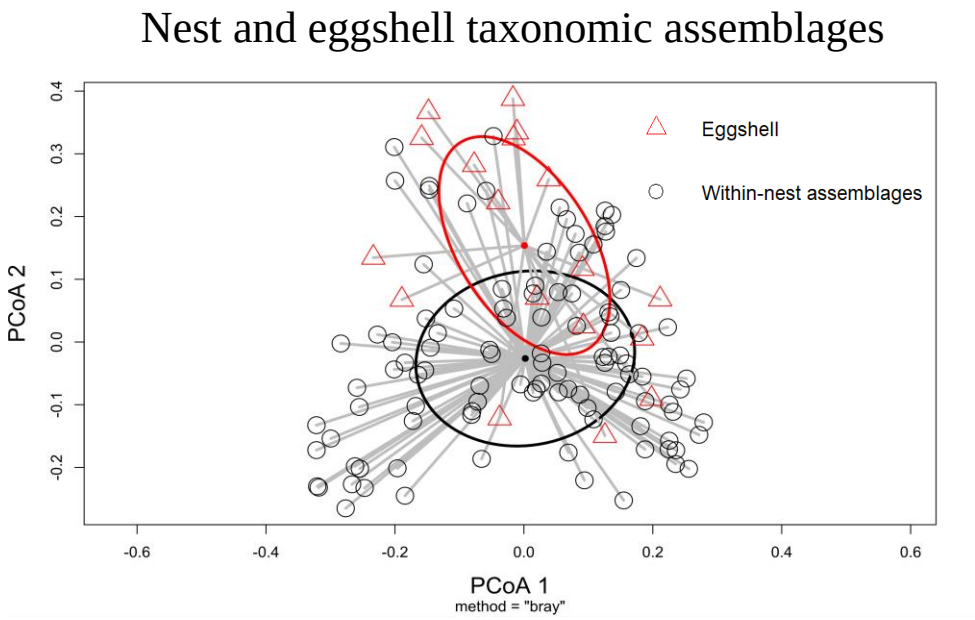

Figure 7: PCoA of within-nest samples (n = 108) and eggshell samples (n = 18) across years. Dud nests (n = 8) and samples collected from the surrounding environment (n = 14) are not included. PERMANOVA indicated that microbial assemblages were statistically different between samples collected from alligator nests and the eggshell ($F_{1,122}$ = 2.425, R^2 = 0.020. p < 0.05).

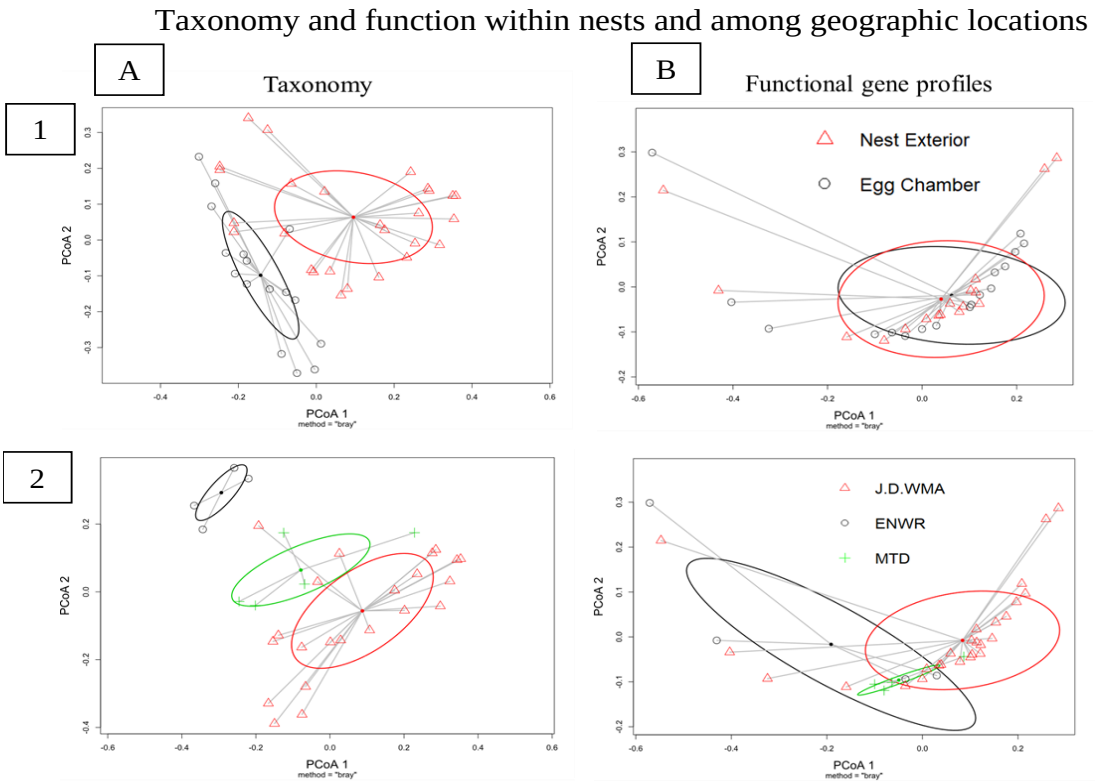

Figure 8: PCoA of taxonomic composition (column A) and functional gene pathways (column B) within nests (row 1, n = 34) and among geograophic sites (row 2, n = 3) . Within-nest assemblages constitute samples collected from the nest exterior surface (sample A, n = 17) and the egg chamber (sample E, n = 17). PERMANOVA indicated that taxonomic assemblages were statistically different within nests ($F_{1, 33}$ =2.456, R^2=0.078 p < 0.05) and among geographic locations ($F_{2, 30}$ =1.924, R^2=0.121, p < 0.05). PERMANOVA indicated redundant functional gene pathways within nests ($F_{1, 33}$ =0.916, R^2=0.028, p > 0.05), yet distinct among geographic sites ($F_{2, 33}$ =2.593, R^2=0.143, p < 0.05).

Appendix B: Tables

Table 1: OTUs indicative of each sample location within alligator nests across geographic sites and years. Indicator value > 0.50, $p < 0.005$. Taxonomy of eggshell indicator taxa are listed in Table 2.

Sample location	Indicative OTU count	*p*-value
Nest exterior surface	31	$p < 0.05$
Eggshell	16	$p < 0.05$
Egg chamber bottom	1	$p < 0.05$
Dripline	182	$p < 0.05$
Surrounding water	194	$p < 0.05$

Table 2: Taxonomic classification of bacteria indicative of the eggshell microbiome.

Indicator value > 0.50, $p < 0.05$.

OTUS	STAT	P.VALUE	PHYLUM	CLASS	ORDER
OTU000293	0.731	0.001	Proteobacteria	Alphaproteobacteria	Caulobacterales
OTU000569	0.72	0.001	Proteobacteria	Deltaproteobacteria	Bdellovibrionales
OTU001324	0.688	0.001	Actinobacteria	Actinobacteria	Micrococcales
OTU002062	0.686	0.001	Actinobacteria	Actinobacteria	Actinobacteria_unclassified
OTU002919	0.663	0.001	Proteobacteria	Deltaproteobacteria	Bdellovibrionales
OTU000043	0.663	0.001	Bacteroidetes	Bacteroidia	Sphingobacteriales
OTU001267	0.631	0.001	Actinobacteria	Actinobacteria	Micrococcales
OTU000190	0.593	0.001	Actinobacteria	Actinobacteria	Micrococcales
OTU002521	0.583	0.001	Bacteroidetes	Bacteroidia	Cytophagales
OTU002222	0.58	0.001	Proteobacteria	Gammaproteobacteria	Xanthomonadales
OTU005748	0.575	0.001	Proteobacteria	Alphaproteobacteria	Caulobacterales
OTU002382	0.556	0.001	Actinobacteria	Actinobacteria	Micrococcales
OTU003182	0.548	0.001	Proteobacteria	Gammaproteobacteria	Xanthomonadales
OTU000734	0.529	0.001	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_unclassified
OTU006888	0.527	0.001	Proteobacteria	Gammaproteobacteria	Xanthomonadales
OTU005627	0.511	0.001	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_unclassified

Table 3: Functional pathways indicative (indicator value > 0.50, $p < 0.05$) of the alligator nest chamber (sample E) which is in direct contact with eggshell surfaces. ****** designates substrate degredation pathways.

EGG CHAMBER FUNCTIONAL PATHWAYS	STAT	P.VALUE
DTDP.L.RHAMNOSE.BIOSYNTHESIS.I	0.845	0.001
SUPERPATHWAY.OF.POLYAMINE.BIOSYNTHESIS.II	0.836	0.002
X2.METHYLCITRATE.CYCLE.I	0.826	0.005
**L.TYROSINE.DEGRADATION.I	0.825	0.007
X2.METHYLCITRATE.CYCLE.II	0.822	0.006
L.ARGININE.BIOSYNTHESIS.III..VIA.N.ACETYL.L.CITRULLINE.	0.79	0.005
SUPERPATHWAY.OF.PYRIMIDINE.RIBONUCLEOTIDES.DE.NOVO.BIOSYNTHESIS	0.782	0.003
**GLUTARYL.COA.DEGRADATION	0.779	0.002
SUPERPATHWAY.OF.FATTY.ACID.BIOSYNTHESIS.INITIATION..E..COLI.	0.777	0.002
X.OCTANOYL..ACYL.CARRIER.PROTEIN..BIOSYNTHESIS..MITOCHONDRIA..YEAST.	0.775	0.002
SUPERPATHWAY.OF.PURINE.NUCLEOTIDES.DE.NOVO.BIOSYNTHESIS.I	0.755	0.003
X5.AMINOIMIDAZOLE.RIBONUCLEOTIDE.BIOSYNTHESIS.I	0.751	0.004
NITRATE.REDUCTION.I..DENITRIFICATION.	0.685	0.004
DTDP.L.RHAMNOSE.BIOSYNTHESIS.I	0.845	0.001
SUPERPATHWAY.OF.POLYAMINE.BIOSYNTHESIS.II	0.836	0.002
X2.METHYLCITRATE.CYCLE.I	0.826	0.005

IACUC APPROVAL

TTU-IACUC-17-18-007

INSTITUTIONAL COMMITTEE FOR THE CARE AND USE OF LABORATORY ANIMALS IN EXPERIMENTATION

COMMITTEE ACTION FORM

Principal Investigator or Activity Director Chris Murray

Campus Address Department of Biology, TTU

College Arts and Sciences Department/Unit Biology

Project Title Microbial assemblages across ecological scales in a crocodilian model system.

The project referenced above has been reviewed. The decision is as follows:

✓ Approved as presented (Date of Approval 02/11/2018)

_____ Approved with stipulations which are: (Date _____)

_____ Not approved for the following reasons: (Date _____)

Signatures:

Committee Chairperson
Dr. Steve Hayslette