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Unearthing the sand microbiome of sea turtle nests with disparate survivorship at a mass-nesting beach in Costa Rica

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ABSTRACT: For endangered sea turtle populations, microbial pathogens of developing embryos are of concern at nesting sites around the globe. For olive ridley turtles, hatching success is markedly lower at mass-nesting sites than at solitary nesting beaches, a case presumably resulting from the abundance of decomposing eggs generated by nesting turtles destroying adjacent eggs. This organic input drives microbial activity, affecting the nest environment (i.e. pO_2 and temperature), and reducing embryo survivorship and hatching success. However, the composition of microbial communities in nest sand has not been studied in detail and the presence of potential pathogens can, therefore, not be discounted. As a part of a larger study that investigated microbial abundance in nests, we employed high-throughput DNA sequencing to compare fungal and bacterial composition in nest sand from areas of disparate embryo survivorship. While we found no differences in alpha-diversity (mean operational taxonomic unit diversity within each site) among nesting areas, the microbial community composition of each area was distinct, and differences in community structure corresponded with variable hatching success. Some sequences of potential sea turtle egg pathogens were obtained (e.g. *Fusarium solani* species complex), but were in low relative abundance, and their presence was not associated with low hatching success. Our results from the arribada beach at Ostional, Costa Rica, provide further evidence that the physical characteristics of the nest (including those that determine microbial composition and activity) are likely more relevant to hatching success than the presence of potential pathogens or microbial community structure alone.

KEY WORDS: Olive ridley · *Lepidochelys olivacea* · Metabarcoding · Microbial diversity · Microbial composition

1. INTRODUCTION

Recent advances in technology have promoted scientific research on microbiomes, allowing us to investigate the species composition of microbial communities associated with a diversity of organisms (Waite et al. 2012, Sarmiento-Ramírez et al. 2014b, Budachetri et al. 2016). This topic is of particular interest in conservation biology, where geographical shifts in distribution resulting from climate change are driving emerging infectious diseases, making them a primary threat to biodiversity (Daszak 2000, Anderson et al. 2004). For sea turtle populations at risk of extinction, there is increasing concern that microbial pathogens are negatively impacting hatching success (Sarmiento-Ramírez et al. 2014a). Herein, we focus on nesting areas of the olive ridley sea turtle *Lepidochelys olivacea* on sandy beaches in Costa Rica.

There are a variety of possible mechanisms by which microbes could impact sea turtle embryo sur-

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vivorship. Studies suggest that most fungi and bacteria likely act as opportunistic pathogens in sea turtle nests, causing the death of embryos already compromised as a result of other factors (e.g. physical and chemical characteristics of the egg and nest sand; Mo et al. 1995, Phillott & Parmenter 2001b, Craven et al. 2007). However, fungi within the *Fusarium solani* species complex have been directly linked with infections and embryo mortality in sea turtles at sites across the globe (Sarmiento-Ramírez et al. 2010, 2014a). Microbes may also indirectly impact embryo survivorship in sea turtles by competing for resources such as oxygen and increasing nest temperature (Bézy et al. 2014). For example, fungal growth can reduce the surface area of the egg available for respiratory gas or heat exchange, which are some of the critical factors for the successful incubation of eggs (Phillott & Parmenter 2001a). Additionally, fungi can deteriorate the calcium in the eggshell and compromise the integrity of the egg, leaving the embryo more susceptible to subsequent fungal invasion (Phillott et al. 2006). Alternatively, cloacal fluid or certain cloacal bacteria and fungi may provide benefits to developing sea turtle embryos by supplying antimicrobial compounds or otherwise inhibiting spore germination of potentially pathogenic species (Keene 2012, Phillott & Parmenter 2012).

While it is the most abundant sea turtle species, eggs of the olive ridley are at particularly high risk of exposure to microbes due to the species' unusual nesting behavior. In addition to nesting solitarily as most sea turtle species do, olive ridley sea turtles also participate in unique synchronous mass-nesting events called arribadas (Spanish for arrivals), during which thousands of turtles emerge from the sea to lay their eggs simultaneously over several consecutive days (Bernardo & Plotkin 2007). With many females arriving to dig their nests and lay their eggs within a relatively small area at the same time, the high density and consequential overlap of nests result in high rates of nest destruction (Cornelius et al. 1991, Ocana et al. 2012). The high density of nests also results in density-dependent competition among eggs for physiological requirements during embryonic development (e.g. sufficient pO_2; Honarvar et al. 2008). Therefore, while a great number of eggs are laid during each mass-nesting event, hatching success is considerably lower at mass-nesting beaches compared to solitary sites (0–32% versus 74–81%, respectively; Cornelius et al. 1991, López-Castro et al. 2004, Valverde et al. 2012, Barrientos-Muñoz et al. 2014, Bézy et al. 2016). However, studies suggest that low hatching success at mass-nesting sites cannot be explained by density-dependent factors alone and other factors are therefore likely at play (Clusella Trullas & Paladino 2007, Valverde et al. 2010, Bézy et al. 2014).

At Ostional, on the Pacific coast of Costa Rica, olive ridley sea turtles nest in both solitary and mass-nesting events throughout the year (Valverde et al. 2012). Hatching success is markedly low at this site (18%), particularly in areas of the beach with high nest densities and during the dry season, when nest temperatures can exceed the lethal limit for embryonic development (Valverde et al. 2010, 2012, Bézy et al. 2014). Low hatching success has been attributed to increased microbial activity due to the high organic matter content of the sand resulting from eggs crushed by other nesting turtles (Cornelius et al. 1991, Clusella Trullas & Paladino 2007, Honarvar et al. 2008, Bézy et al. 2014). Higher microbial metabolic activity increases nest temperatures and decreases the availability of oxygen to egg clutches (Bézy et al. 2014, 2015). However, the presence of microbial pathogens and/or opportunists could also result in embryo death and contribute to low hatching success in sea turtle nests at Ostional, as has been reported at other sites (Wyneken et al. 1988, Phillott & Parmenter 2001b, Sarmiento-Ramírez et al. 2010).

Despite the possible threat of pathogenic and other biological interactions of microbes with sea turtle eggs, our understanding of the impact microbial communities may have on sea turtle embryo survivorship remains limited. While numerous studies have identified microbes in association with sea turtle nests (Wyneken et al. 1988, Phillott et al. 2001, Phillott et al. 2004, Craven et al. 2007, Zieger et al. 2009, Güçlü et al. 2010, Sarmiento-Ramírez et al. 2010, 2017, Al-Bahry et al. 2011, Soslau et al. 2011, Keene et al. 2014, Sidique et al. 2017, Candan 2018), fewer have investigated the interaction between microbes and sea turtles within the natural nest environment (Honarvar et al. 2011, Patino-Martinez et al. 2012, Bézy et al. 2014, 2015, Sarmiento-Ramírez et al. 2014a). Consequently, as part of a larger study, we used high-throughput DNA sequencing to characterize the diversity and relative abundance of fungi and bacteria in *in situ* sea turtle nest sand at Ostional, Costa Rica. Here, we incorporate the composition of microbes identified in the present study with previously published data on the microbial abundance, environmental parameters, and hatching success of these same nests (Bézy et al. 2014) to better understand the role microbes may play in sea turtle embryo survivorship at mass-nesting beaches.

2. MATERIALS AND METHODS

2.1. Study site and sand sampling

Nest sand samples were collected at the Ostional National Wildlife Refuge in August 2012 as part of a larger study (Bézy et al. 2014), which was approved by the College of Charleston Institutional Animal Care and Use Committee (IACUC) and conducted under permits granted by the government of Costa Rica (ACT-OR-DR-078 and R-020-2012-OT-CONAGEBIO). The refuge is located on the Pacific coast of the Nicoya Peninsula in Costa Rica (Fig. 1; Bézy et al. 2014). We randomly selected 5 nests laid above the high-tide line, on the same night of a mass-nesting event, in 3 different areas within a 500-m stretch of beach (Fig. 1). The entire duration of oviposition was observed to count the number of eggs laid. After oviposition concluded, a 5-sided 50 × 50 × 15 cm (width × length × height) wire mesh cage with a wooden frame and a mesh top was placed over the nest and buried entirely below the surface of the sand to prevent depredation and nest destruction by subsequently nesting females. The beach areas were categorized using characteristics such as nesting density and tidal wash (Table 1; Bézy et al. 2014). The low-density nesting area was distinguished as an area where a lower density of females nest during the arribada; the tidal-wash area was a supratidal area where a lower density of females nest during the arribada and where the tide occasionally washed over into the adjacent estuary, resulting in frequent turnover of sand; and the high-density nesting area was a portion of the beach where nesting tended to concentrate during arribada events.

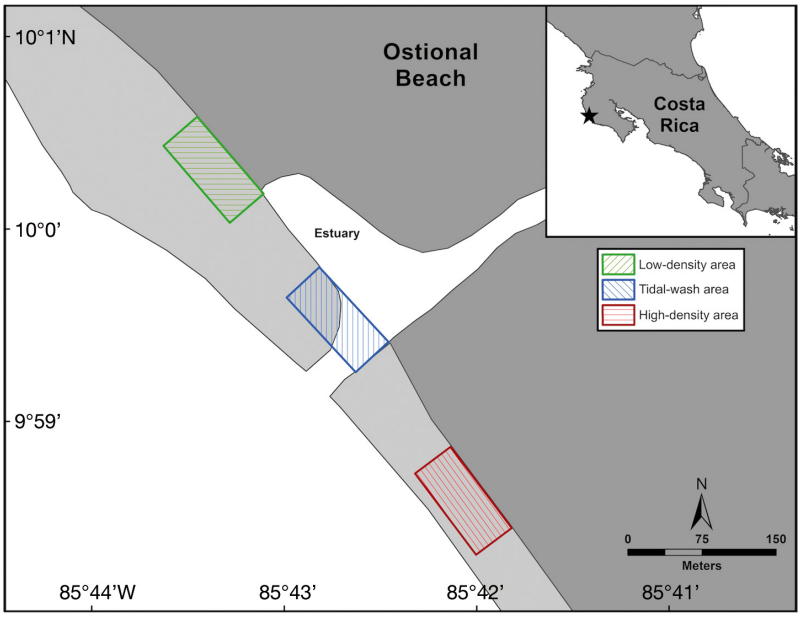

Fig. 1. Map of Ostional, Costa Rica, with the sampling areas indicated

Oxygen and temperature data were collected from the center of each nest chamber throughout the incubation period and were reported previously (Bézy et al. 2014). Briefly, nest pO_2 was measured using a flow-through oxygen sensor (S108 Oxygen Analyzer, Qubit Systems) and temperature data were collected using temperature dataloggers (HOBO pendant®, Onset

Table 1. Characteristics of the sampling areas. Average nest conditions and sand characteristics measured for each nesting area as a part of a larger study (Bézy et al. 2014). Different letters denote statistical difference. Symbols denote level of significance (*p < 0.05, **p < 0.001, ***p < 0.0001)

	Low density		Tidal wash		High density	
Hatching success (%)	68.20	A	54.70	A	0.60	B***
Nest density (m^{-2})	3	A	2	A	5.2	B*
Nest temperature (°C)[1]	33.4	A	31.8	B**	34.3	A
Nest pO_2 (kPa)[a]	18.4	A	18.4	A	13.4	B***
Bacterial abundance[b]	1.9×10^8	A	5.8×10^8	A	6.1×10^9	B*
Fungal abundance[b]	8.4×10^8	A	3.9×10^9	A	1.2×10^{10}	B.
Organic matter content (%)	3.10	A	2.70	A	3.70	B*
Mean grain size (ϕ)	1.45	A*	1.82	B	1.82	B
Quartile deviation (σ_ϕ)	0.98		1.01		1	
Grain skewness (Sk_ϕ)	1.03		0.36		0.35	
Elevation (cm)	20.5	A	158	B***	41.4	A

[a]Average over the entire incubation period; [b]16S or 18S copy number g^{-1} of nest sand

Computer Corporation). At the end of the incubation period, approximately 15 g of sand was collected from the center of each nest chamber (~30 cm depth) directly into sterile collection tubes and stored at −20°C until analysis. Granulometric characteristics, organic matter content, and microbial abundance of nest sand are reported in Bézy et al. (2014). Briefly, samples of dried sand were fractionated with a set of sieves to determine the particle size distribution by mass and calculate the median grain size (ϕ), quartile deviation (QD), and skewness (*Sk*). The organic matter content of nest sand was determined as the percent loss of mass after dry combustion (loss-on-ignition method). The microbial abundance of nest sand was quantified as the 16S and 18S copy number g^{-1} of nest sand as determined by a qPCR analysis using universal primers for bacteria and fungi, respectively. Nests were excavated 3 d after the first emergence of hatchlings to quantify hatching success by recording the total number of hatchlings that hatched out of their eggshell relative to the total number of eggs originally deposited in the nest. The wire mesh cage surrounding the nest prevented the superimposition of other nests during the arribada and permitted us to distinguish the nest from those of a previous arribada based on the coloration and rigidity of the eggshells (Bézy et al. 2016).

2.2. DNA extraction

Samples were thawed and homogenized prior to subsampling for DNA extraction using a PowerSoil DNA Isolation Kit (MO BIO Laboratories). DNA isolation was performed according to the manufacturer's instructions with the following modifications to increase DNA yields, according to Bézy et al. (2014): 1 g of the sample was added to the PowerBead Tubes, samples were subjected to 5 min at approximately 2000 oscillations min^{-1} in a bead beater (Mini Beadbeater-8, Biospec Products), and 3 freeze–thaw cycles were performed during the lysis step (−20°C and 70°C for 30 min each).

2.3. High-throughput DNA sequencing

An amplicon library containing both fungi and bacteria amplicons was produced following the Ion Torrent Amplicon Library Preparation protocol (Fusion Method, Life Technologies). For fungi, primers fITS7 (5′-GTG ART CAT CGA ATC TTT G-3′; Ihrmark et al. 2012) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; White et al. 1990) were used to amplify the second internal transcribed spacer region (ITS2) of small subunit ribosomal DNA (rDNA), which is highly variable at the species level (Nilsson et al. 2008). Primers U515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and U926R (5′-CCG TCA ATT CMT TTR AGT-3′) were used to amplify a portion of bacterial 16S rDNA containing variable regions 4 and 5 (Fujimoto et al. 2014). Forward and reverse primers included a 5′ tail for Ion Torrent sequencing (adapter A: 5′-CCA TCT CAT CCC TGC GTG TCT CCG AC-3′, adapter TrP1: 5′-CCT CTC TAT GGG CAG TCG GTG AT-3′, respectively). The suite of forward primers also contained the 4-base pair (bp) library key, an individual barcode for each sample (Ion Xpress Barcode Adapters 001, 003-011, 013-015, 022-029, 032-036; Life Technologies), and a barcode adapter.

For both assays, a 50 µl total volume PCR contained 0.2 µM of each primer, 1× Q5 Hot Start High Fidelity Master Mix (New England Biolabs), and 5 µl DNA template. The cycling conditions were as follows: an initial denaturation at 98°C for 5 min was followed by 32 cycles of 30 s at 98°C, 30 s at 57°C and 1 min at 72°C and a final extension of 72°C for 4 min. PCR products were electrophoresed and visualized on 1% agarose gels using GelRed (Biotium) and UV transillumination. For bacteria, duplicate reactions were pooled and subsequently gel-extracted using a QIAquick Gel Extraction kit (Qiagen). Single reactions were performed to produce the fungal products, which were purified using 0.8× Agencourt AMPure XP reagent (Beckman Coulter). Each individual fungal and bacterial library (e.g. from a single sample) was quantified using the DNA 1000 Kit and a Bioanalyzer 2100 (Agilent) following the manufacturer's instructions. An equimolar solution of all individual libraries was prepared and was also quantified using the DNA 1000 Kit and a Bioanalyzer 2100 (Agilent). The final library was further prepared at the Medical University of South Carolina's Proteogenomics Facility (Charleston, SC, USA) and sequenced on an Ion Torrent Personal Genome Machine using an Ion 314 Chip Kit v2 (Life Technologies).

2.4. Data analysis

Sequencing data were deposited with links to BioProject accession number PRJNA588456 in the NCBI BioProject database (Leinonen et al. 2011; https://www.ncbi.nlm.nih.gov/bioproject/). QIIME (Quantitative Insights Into Microbial Ecology; Caporaso et

al. 2010), an open-source bioinformatics pipeline, was employed to analyze the Ion Torrent sequencing data. Raw sequences were filtered based on size and quality scores: we removed any sequences less than 300 bp, greater than 600 bp, and those with average quality scores less than 20. USEARCH v.5.2.236 (Edgar 2010) was used to detect and remove chimeras using UCHIME (Edgar et al. 2011), remove singletons, and assign operational taxonomic units (OTUs; sequence similarity = 97%). Representative sequences were chosen and compared to either the Greengenes bacterial 16S rDNA database (Werner et al. 2012, McDonald et al. 2012) or the UNITE fungal ITS rDNA database (Kõljalg et al. 2005, 2013), using the RDP classifier (Wang et al. 2007) to assign taxonomy to each representative sequence. A 97% clustering identity was used for bacteria, and we used the dynamic taxonomy and reference sequence files for clustering identity for fungi given the differing species delimitations depending on fungal lineage. OTU tables were generated and rarefied (subsampled) for subsequent analyses based on the sample with the fewest sequences. Shannon diversity (H') and Chao 1 richness indices, the number of observed OTUs (S), and Heip's evenness (E) were calculated in QIIME. We also generated diversity metric rarefaction curves for both bacteria and fungi to evaluate if the sequencing depth sufficiently captured the overall composition of each sample. We report taxonomy to family unless this level of resolution was not available in the taxonomic assignment database, in which case we report the lowest taxonomic level available. Representative sequences from families of interest (i.e. those that include potential known pathogens or previously have been identified in association with failed nests) were explored to species level. If species-level data were not available in the taxonomic assignment databases, we compared these sequences to those in the GenBank database using the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990); alignments and p-distance calculations were done using MEGA7 (Kumar et al. 2016).

Sequences resulting from the bacterial library were aligned using PyNAST (Caporaso et al. 2010), filtered (gap filter threshold = 0.8; entropy threshold = 0.10), and an approximately maximum-likelihood phylogenetic tree was generated using FastTree 2.1.3 (Price et al. 2010) in order to generate weighted and unweighted UniFrac distance matrices (Lozupone & Knight 2005) in QIIME. Bray-Curtis distance matrices were also generated for comparison. For fungi, Bray-Curtis and Euclidean distance matrices were generated. Analysis of similarity (ANOSIM; Clarke 1993) with 1000 permutations was used to test for differences in communities among sampling sites. ANOSIM R-values were interpreted as in Clarke & Gorley (2001): $R > 0.75$ = well separated; $R > 0.5$ = overlapping, but different; $R > 0.25$ = barely separable; and $R < 0.25$ = indistinguishable. Results of all distance statistics to quantify the compositional dissimilarity across nesting areas were qualitatively similar, and we therefore present only Bray-Curtis dissimilarities here. Principal component analyses (PCAs) were performed in JMP Pro 12 (SAS Institute Inc.). Similarity percentages (SIMPER; Clarke 1993) analysis was done using PRIMER-E v6 (Clarke & Gorley 2006) to determine a given family's contribution to any observed dissimilarity between sites, and Spearman's correlations, done in JMP (SAS Institute), were used to examine the relationship between the relative abundance of the distinguishing families and hatching success. Distance matrices of the environmental data collected (e.g. granulometric data, pO_2, temperature) were generated in QIIME, and Mantel tests with 1000 permutations were performed to determine if there were significant correlations between community dissimilarity and differences in hatching success and environmental factors among nesting areas.

All other statistics were performed using JMP Pro 12 (SAS Institute Inc.). The Shapiro-Wilk test was used to check for normality, and the Levene test was used to test for unequal variances. If data were normally distributed and had equal variance, ANOVA and subsequent Tukey's honestly significant difference (HSD) pairwise tests were performed. If data and transformations of these data were found to be non-parametric ($p < 0.05$), Kruskal-Wallis tests and subsequent non-parametric comparisons using the Wilcoxon method for each pair were performed.

3. RESULTS

3.1. Sand sampling

Samples from 2 nests were excluded from the analysis due to unforeseeable factors that likely caused a change in the variables that we attempted to control in the previous study (Bézy et al. 2014): one nest in the high-density area was partially shaded by a palm tree, and a nest in the tidal-wash area became inundated during the incubation period. Conditions and sand characteristics of the nests from this study were previously described in Bézy et al. (2014). Briefly, nests in the high-density area had signifi-

cantly lower hatching success and experienced higher nest densities than nests in other areas of the beach (Table 1). Nest temperature was lower in nests located in the tidal-wash area (Table 1). In the high-density area of the beach, nest pO_2 was significantly lower and the bacterial abundance and organic matter content of nest sand were higher than in other areas of the beach (Table 1). Mean grain size was larger in the low-density area of the beach, and the tidal-wash area was at a significantly higher elevation than the other areas of the beach (Table 1).

3.2. High-throughput DNA sequencing

Ion Torrent sequencing produced 573 037 total reads with a mean read length of 407 bp. After quality filtering, the bacterial 16S rDNA library contained a total of 25 980 sequences with a mean ± SD of 1998 ± 1104 sequences per library, a range of 562 to 4463 sequences per library (rarefaction depth = 562), and a total of 2243 OTUs. The fungal ITS rDNA library contained a total of 91 716 sequences with a mean of 7055 ± 2998 sequences per library, a range of 971 to 11 145 sequences per library, and a total of 1613 OTUs. A low-density nest, which had the fewest number of sequences (rarefaction depth = 971), was excluded for subsequent fungal alpha- and beta-diversity analyses, because we had excluded one nest from both of the other areas (see above) and doing so also allowed us to rarefy at a greater depth (rarefaction depth = 3715). Removing this nest resulted in a total of 1497 OTUs in the fungal library. Rarefaction curves for fungal diversity metrics indicated sufficient sampling (Fig. S1 in the Supplement at www-int-res.com/articles/suppl/a085p071_supp.pdf). For bacteria, while Shannon and evenness rarefaction curves plateaued, Chao 1 and observed OTU curves were not saturated (Fig. S2), thus richness calculations may be underestimated (Hughes et al. 2001).

3.3. Microbial diversity

There was no significant difference in any of the diversity metrics we considered with respect to nesting area (ANOVA, $p > 0.05$; Table 2). However, fungal and bacterial community structure did vary among nesting areas (ANOSIM, R = 0.558, p = 0.001, and R = 0.785, p = 0.001, respectively; Fig. 2). Fungal communities from the high- and low-density areas were completely separate (ANOSIM, R = 1.00, p = 0.021), but fungal communities in the tidal-wash area overlapped with both the low- and high-density nesting areas (ANOSIM, R = 0.729, p = 0.034, and R = 0.440, p = 0.032, respectively). Sixty-six percent of fungal OTUs (990 sequences) were unique to a single nesting area, whereas under 6% were common to all 3 nesting areas (Fig. 3A). Bacterial communities were also well separated between areas (ANOSIM, R = 0.558, p = 0.001): communities from the high-density area were different from both the low-density and tidal-wash areas (ANOSIM, R = 0.697, p = 0.011, and R = 0.807, p = 0.027, respectively), while those from the tidal-wash and low-density areas were less distinct (ANOSIM, R = 0.250, p = 0.050). Sixty-nine percent of bacterial OTUs were unique to a single nesting area, while 31% were common to at least 2 areas; just 7% were common to all 3 nesting areas (Fig. 3B).

3.4. Fungal community composition

Fungal communities in sand from all areas were predominantly composed of fungi from the phylum Ascomycota (Table 3). Nectriaceae, the family of fungi that includes the *Fusarium solani* species complex, was present in all nesting areas, but at a small relative abundance (0.05, high density; 0.12, tidal wash; and 0.27%, low density). Additionally, this family did not contribute greatly to average dissimilarity across beach areas (0.30 – 0.63% dissimilarity). The sequences found within this family were most

Table 2. Diversity metrics for fungi and bacteria in nest sand located in different areas of the beach: low-density nesting area, tidal-wash area, and high-density nesting area. Shannon diversity and Chao 1 richness indices, the number of observed operational taxonomic units (OTUs), and Heip's evenness were calculated in QIIME. There was no significant difference in any diversity metric among nesting areas ($p > 0.05$). Data are means ± SEM

	Low density	Tidal wash	High density
Fungi			
Diversity	5.12 ± 0.23	5.64 ± 0.34	5.65 ± 0.19
Observed OTUs	315.20 ± 20.25	301.23 ± 45.82	295.78 ± 10.99
Evenness	0.11 ± 0.01	0.17 ± 0.02	0.17 ± 0.03
Richness	430.78 ± 28.39	411.91 ± 64.71	404.70 ± 16.79
Bacteria			
Diversity	7.36 ± 0.09	7.30 ± 0.44	6.43 ± 0.63
Observed OTUs	256.52 ± 6.49	265.63 ± 34.35	212.12 ± 30.17
Evenness	0.64 ± 0.03	0.63 ± 0.08	0.46 ± 0.09
Richness	544.47 ± 30.86	610.92 ± 89.99	475.52 ± 71.06

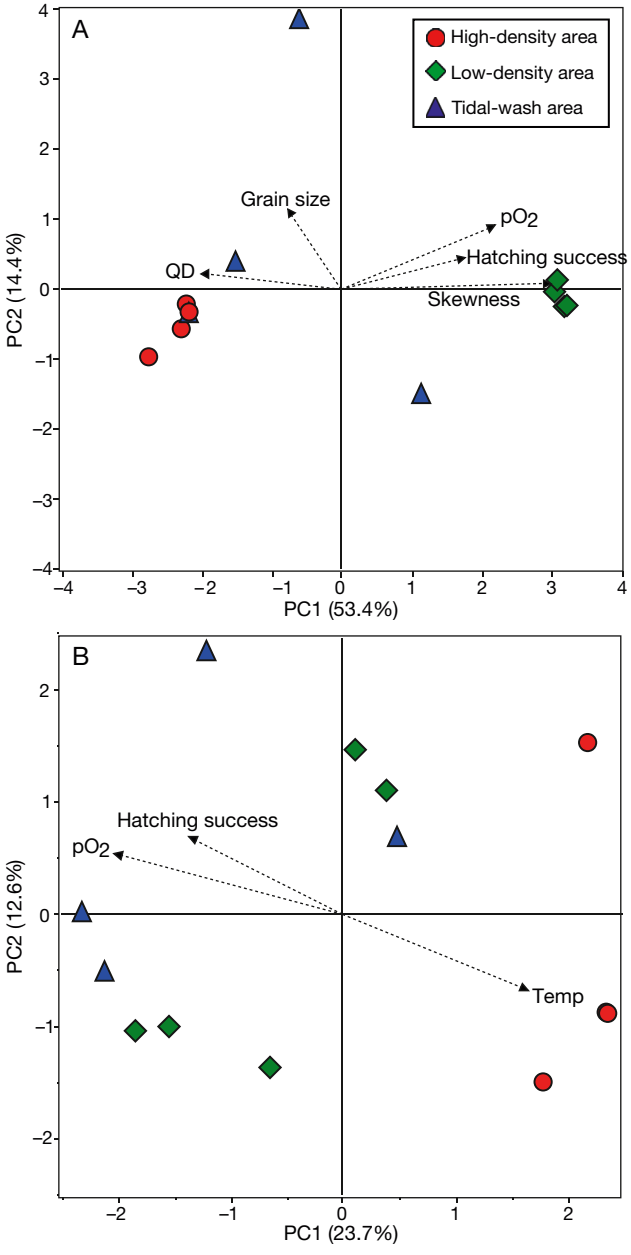

Fig. 2. Principal components analysis (PCA) plots based on Bray-Curtis dissimilarity displaying nesting areas and significant ($p < 0.05$) environmental vectors based on Mantel tests for (A) fungal and (B) bacterial community structure. Plots were generated in JMP Pro version 14 (SAS Institute) and edited in Inkscape 1.0 (www.inkscape.org/). QD: quartile deviation

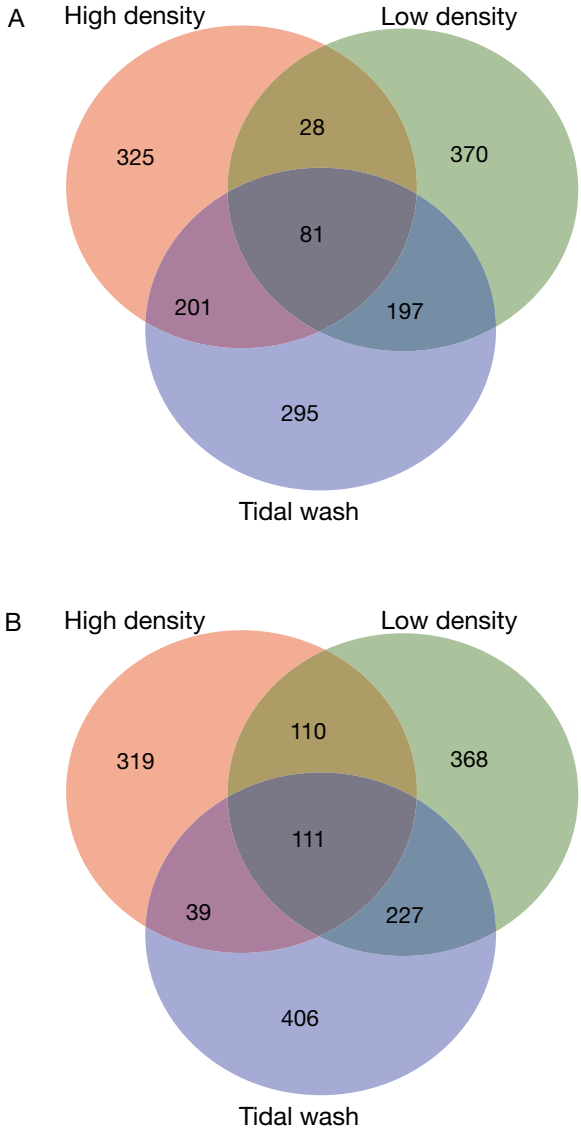

Fig. 3. Number of shared and unique operational taxonomic units (OTUs) for (A) fungi and (B) bacteria among nesting areas

closely related to *F. fujikuroi*, *F. keratoplasticum*, and other undescribed/unknown *Fusarium* spp.

There was a higher relative abundance of Gymnoascaceae in the high-density and tidal-wash areas compared to the low-density area, with this group contributing to 13.97% and 8.68% average dissimilarity, respectively (Table 3, Table S1). The high-density and tidal-wash areas were 59.59% dissimilar, with Chaetomiaceae contributing the most difference (6.79%). Mycosphaerellaceae was more abundant in the tidal-wash area (4.10% and 4.99% dissimilarity with the low- and high-density areas, respectively; Table S1). However, this family was present at one order of magnitude greater relative abundance in one nest in the tidal-wash area in comparison to all others in this area. The tidal-wash area also had a greater relative abundance of Dothideales (ANOVA, $p < 0.05$; Table 3). Overall, the high-density and tidal-wash areas were 67.81% and 69.57% dis-

Table 3. Summary of the fungal composition from nests located in different areas of the beach: low-density nesting area, tidal-wash area, and high-density nesting area. Only taxa with more than 1% relative abundance (at any one area) are shown here due to the great number of taxa. Taxonomy is reported to family unless this level of resolution was not available in the taxonomic assignment database, in which case the lowest taxonomic level available is reported. Different letters denote statistical difference ($p < 0.05$) of pairwise comparisons. **Bold** denotes families that typify a site (>2% average SIMPER dissimilarity)

Group		Fungal composition (%)					
		Low density		Tidal wash		High density	
Ascomycota	Other	0.40		**2.23**		**7.09**	
	Capnodiales; other	0.33	A,B	1.57	A	0.00	B
	Chaetomiaceae	**74.56**	A	11.20	B	**18.71**	B
	Dothideales; incertae sedis	0.01	B	1.66	A	0.01	B
	Dothideomycetes; other	0.02		1.46		0.01	
	Gymnoascaceae	**1.21**	A	**26.68**	A,B	**41.85**	B
	Herpotrichiellaceae	1.05		0.06		0.44	
	Hypocreales; incertae sedis	**3.72**		1.00		0.25	
	Microascaceae	0.87		**11.61**		**5.62**	
	Mycosphaerellaceae	1.66	A,B	**9.16**	A	0.05	B
	Myrmecridiaceae	1.10		0.00		0.00	
	Onygenaceae	1.28		0.13		0.42	
	Onygenales; other	0.17		2.63		**2.74**	
	Pleosporaceae	0.23		4.57		0.05	
	Pleosporales; other	0.01	A	0.16	A,B	1.60	B
	Saccharomycetales	0.03		1.38		0.02	
	Sordariaceae	2.64		0.00		0.00	
	Trichocomaceae	4.76		0.96		0.49	
	Trichosphaeriaceae	0.49		2.34		0.00	
	Xylariaceae	0.12		1.63		0.00	
Basidiomycota	Cystobasidiaceae	0.01		0.00		5.68	
	Lyophyllaceae	0.00		2.03		0.00	
Mortierellomycota	Mortierellaceae	0.15		6.78		**7.23**	
Fungi	Other	0.39		**2.15**		1.77	

similar with the low-density area, respectively. Chaetomiaceae was present in the greatest relative abundance in the low-density area (74.56%), differentiating it from both the high-density and tidal-wash areas (12.34% and 13.94% dissimilarity, respectively). The low-density area also had a lower relative abundance of Microascaceae than the other areas (5.01% and 3.71% dissimilarity with the tidal-wash and high-density areas, respectively; Table S1). Of the fungal taxa responsible for differentiating nesting areas, only the Gymnoasceae were significantly correlated with hatching success ($\rho = -0.599$, $p = 0.040$; Table S3).

3.5. Bacterial community composition

Bacillaceae was the most abundant bacterial group in all 3 nesting areas (Table 4). Vibrionaceae differentiated the tidal-wash area from both the low- and high-density areas (contributing 2.52% and 2.19% to average dissimilarity, respectively; Table S2), but data were influenced by the high relative abundance (34.7%) of this family in one nest only; relative abundances in the high- and low-density areas were only 0.09% and 0.11%, respectively. The high-density area had a greater relative abundance of Moraxellaceae, Exiguobacteraceae, and Thermonosporaceae compared to both the low-density and tidal-wash areas (Table 4). However, Moraxellaceae sequences were only present in one nest from the high-density area. The high-density area also had a higher relative abundance of Gaiellaceae compared to the tidal-wash area (1.73% dissimilarity). Relative abundance of the Gaiellaceae family was negatively correlated to hatching success ($\rho = -0.599$, $p = 0.031$), whereas the Gitt-GS-136 clade was positively correlated ($\rho = 0.580$, $p = 0.038$; Table S4).

3.6. Correlations with nest characteristics

Fungal community composition dissimilarity was correlated with differences in granulometric characteristics, nest pO_2, and hatching success (Fig. 2, Table 5). Bacterial community dissimilarity was also corre-

Table 4. Summary of the bacterial composition from nests located in different areas of the beach; the low-density nesting area, the tidal-wash area, and the high-density nesting area. Only taxa with greater than 2% relative abundance (at any one area) are shown here due to the great number of taxa. Taxonomy is reported to family unless this level of resolution was not available in the taxonomic assignment database, in which case the lowest taxonomic level available is reported. Different letters denote statistical difference ($p < 0.05$) of pairwise comparisons. **Bold** denotes families that typify a site (>2% average SIMPER dissimilarity)

Group		Bacterial composition (%)					
		Low density		Tidal wash		High density	
Actinobacteria	Acidimicrobiales	2.88		1.47		0.40	
	Gaiellaceae	2.88	A,B	2.00	A	**8.27**	B
	Nocardioidaceae	2.49		4.36		0.93	
	Thermomonosporaceae	0.32	A	0.09	A	3.91	B
Chloroflexi	Ellin6529	2.24	A	1.16	A,B	0.49	B
	Gitt-GS-136	**4.13**	A	3.56	A,B	0.98	B
Firmicutes	Bacillales	2.35		1.20		1.11	
	Bacillaceae	**16.05**		**12.86**		**16.28**	
	Exiguobacteraceae	0		0		5.56	
	Paenibacillaceae	3.17		4.40		2.85	
	Planococcaceae	3.31		0.76		4.09	
	Tissierellaceae	0.96		0.58		2.67	
Planctomycetes	Pirellulaceae	**4.59**		**7.25**		1.82	
Proteobacteria	Hyphomicrobiaceae	3.02	A,B	0.44	A	**3.91**	B
	Betaproteobacteria MND1	2.70	A	2.58	A,B	0.22	B
	Moraxellaceae	0.04		0.04		11.30	
	Rhodospirillales	1.39		2.09		1.11	
	Vibrionaceae	0.11		8.67		0.09	

Table 5. Mantel test results showing correlations between the microbial composition of nest sand (Bray-Curtis distance) and differences in hatching success and in the physicochemical characteristics of the nest (NS: not significant, $p > 0.05$)

	Fungi		Bacteria	
	R	p	R	p
Hatching success	0.461	0.006	0.438	0.002
Nest temperature[a]	NS	NS	0.303	0.02
Nest pO_2[a]	0.398	0.003	0.442	0.004
Organic matter content	NS	NS	NS	NS
Mean grain size	0.623	0.001	NS	NS
Quartile deviation	0.515	0.003	NS	NS
Grain skewness	0.593	0.001	NS	NS

[a]Average over the entire incubation period

lated with nest pO_2 and hatching success, in addition to nest temperature (Fig. 2, Table 5).

4. DISCUSSION

As a part of a larger study that investigated microbial abundance, physicochemical characteristics, and hatching success of sea turtle nests at a mass-nesting beach (Bézy et al. 2014), we used high-throughput DNA metabarcoding to examine fungal and bacterial communities in nest sand samples from areas of disparate hatching success. While there was no difference in microbial alpha-diversity among nesting areas, the microbial community compositions of low-density, high-density, and tidal-wash areas of the beach were distinct, which indicates a connection between microbial community structure and embryo survivorship of sea turtles.

The comparison of microbial diversity across areas of disparate hatching success under relatively controlled conditions in this study provides a holistic *in situ* overview of taxa in association with sea turtle nests in Ostional. Although we were unable to pinpoint specific microbes that appeared to affect (positively or negatively) sea turtle embryo survivorship, differences in hatching success were correlated with microbial composition dissimilarity, indicating that microbial community structure likely has a direct, or at least an indirect, relationship with embryo survivorship. While some of the microbial groups identified contain known pathogens, these were unequally distributed, found in low relative abundance overall, and were not disparate in relative abundance among nesting areas. The presence of potential pathogens, therefore, appears not to have been a primary factor affecting embryo mortality in nests within the high-density area where hatching success was lowest.

Instead, our results suggest that microbial composition in sea turtle nest sand corresponds to particular environmental conditions and that an interaction of these factors is more relevant to determining hatching success than the presence of pathogenic groups alone. Overall, the results are in agreement with previous observations that implicated changes in nest environment (i.e. nest pO_2 and temperature), due to high fungal and bacterial abundance, as the detrimental effect on hatching success (Bézy et al. 2014).

The family of fungi that includes the *Fusarium solani* species complex (Nectriaceae), a known pathogen to sea turtle eggs, was identified in all nesting areas at a low relative abundance and did not contribute to differences in community structure across beach areas. Because the development of disease from *F. solani* is promoted by tidal inundation and high clay or silt content of nest sand (Sarmiento-Ramírez et al. 2014a), we expected sequences of this pathogen to contribute to the dissimilarity between sites with differences in tidal inundation and grain size. However, the relative abundance of *Fusarium* was <1% across all beach areas in our study. Therefore, the results suggest that tidal inundation and grain size do not greatly influence the abundance of *Fusarium* at Ostional. While strong evidence exists for a pathogenic role of the *Fusarium solani* species complex on sea turtle embryos (Sarmiento-Ramírez et al. 2010), these fungi have also been observed in viable eggs and in cutaneous infections in sea turtles, suggesting that microclimate or immunosuppression likely play a role in the pathogenic nature of this species complex (Castellá et al. 1999, Sarmiento-Ramírez et al. 2014a).

Several of the other microbial families identified in the sea turtle nest sand may contain potentially pathogenic or opportunist species; however, these were not disparate in relative abundance among nesting areas and were present in low relative abundance overall. For example, the area most frequently exposed to tidal exchange had the highest relative abundance of Vibrionaceae. This group has been identified at Ostional and other sea turtle nesting beaches (Wyneken et al. 1988, Acuña et al. 1999, Awong-Taylor et al. 2008), and contains several pathogenic species, including *Vibrio mimicus.* However, *Vibrio* spp. sequences were present at low relative abundances, with *Photobacterium* and uncultured species sequences comprising the majority of Vibrionaceae sequences (5.5%, tidal wash; 0%, high and low density). Our results also suggest that the *Vibrio* spp. sequences we did obtain were not likely to be from *V. mimicus*. Additionally, *Acinetobacter* spp. (Moraxellaceae) have been identified as potentially opportunistic pathogens associated with sea turtle nests in previous studies (Wyneken et al. 1988, Mo et al. 1990, Awong-Taylor et al. 2008, Keene 2012, Patino-Martinez et al. 2012, Keene et al. 2014), but sequences from this group were present at a high relative abundance in only one nest in the high-density area and at a low relative abundance overall (0.7%, high density; 0%, low density and tidal wash). Likewise, sequences from the genus *Allescheria* (syn. *Scedosporium*, *Monascus*) (Microascaceae), a fungus previously identified in association with sea turtle eggs at an arribada site (Mo et al. 1990), was present but found in low relative abundance across all areas (1.1%, high density; 2.0%, tidal wash; and 0.6%, low density).

We did find that fungal and bacterial community dissimilarities were correlated with differences in hatching success. However, confident assignment of cause and effect is challenging given that hatching success covaried with environmental parameters, namely pO_2 and temperature. Likewise, relative abundances of a few microbial groups were correlated with hatching success, although causal relationships are doubtful. For instance, although abundance of the Gymnoascaceae was negatively correlated with hatching success, this fungal family was found in high relative abundance in both the high-density and tidal-wash nesting areas, the latter of which exhibited very high (~55%) hatching success. Among the bacteria, abundance of Gaiellaceae was likewise negatively correlated with hatching success. Pathogens among this group are unknown, but this family is known to thrive under conditions of high organic matter (Lin et al. 2019, Zhang et al. 2019). Thus, the negative correlation with hatching success may be due to co-variation with sand organic levels. In contrast, the Gitt-GS-136 clade was positively associated with hatching success. However, no beneficial relationships between Gitt-GS-136 and animals are known; therefore, we can only speculate as to whether these bacteria directly enhance hatching success in some way or the correlation is again due to co-variation with some other variable (e.g. pO_2).

The microbial composition of nest sand at Ostional is therefore likely predicated by the environmental conditions and physical characteristics of the nest sand in different areas of the beach (e.g. grain size, organic matter), where these conditions might favor the growth of certain microbial groups, allowing distinct families to dominate. At another mass-nesting site in Costa Rica, distinct bacterial communities

were also present in areas of the beach with different nesting densities and tidal exposure (i.e. high, middle, and low tidal zones; Honarvar et al. 2011). In the present study, correlations were found between fungal and bacterial community dissimilarity and several physical characteristics of the nests (Table 5). The results suggest fungal community composition is more closely linked with granulometric characteristics while bacterial communities appear to be more strongly correlated with temperature, pO_2, and possibly other environmental conditions that we did not measure (e.g. salinity, substrate water potential). Previous studies suggest that fungi and bacteria likely act as opportunistic agents when the embryo is compromised due to other physical and environmental stressors (Mo et al. 1995, Sarmiento-Ramírez et al. 2014a). Certain environmental conditions of nest sand may therefore favor these opportunistic agents while others favor microbial groups that are neutral or beneficial to embryonic development by outcompeting or otherwise preventing pathogenic or opportunistic species from colonizing. For example, Vibrionaceae sequences were dominant in the tidal-wash area, where frequent exposure to the tides likely predisposes an elevated abundance of halophilic vibrios, which can represent up to 40% of bacterial communities in seawater (Chan et al. 1986). Alternatively, microbes may also be introduced into the nest and sand by the mother and persist only in environments with suitable conditions for microbial growth (Mo et al. 1990, Phillott et al. 2002, Keene 2012, Keene et al. 2014, Gleason et al. 2020).

Our findings support the prospective use of microbiomes as bioindicators. For example, the second-most abundant bacterial family in the high-density area (Gaiellaceae) is composed of strict aerobes and chemoorganotrophs associated with plants, and its presence is used as an indicator of low carbon-to-nitrogen (C:N) ratios in sediment (Hermans et al. 2016). Our study site presents an extreme case, where the high organic matter content of the sand in areas of the beach with high nest densities (Bézy et al. 2014) likely makes the nest sand more similar to that of terrestrial sources than the typical sea turtle nesting beach. The presence of decaying sea turtle eggs might disproportionately raise the level of nitrogen in nest sand, lowering the C:N ratio and favoring the growth of Gaiellaceae. Therefore, the microbiome of the nest may serve as an indicator of nest conditions that predispose a nest to failure or success regardless of the presence of particular microbes.

Overall, this work represents an important step towards improving our understanding of the impact of microbial community structure on sea turtle embryo survivorship at this location, with potential applications to conservation. Future studies should investigate management practices that could be applied *in situ* and in hatcheries to manipulate the microbiome of nest substrate and increase hatching success.

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