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Fecal and cloacal microbiomes of cold-stunned loggerhead *Caretta caretta*, Kemp's ridley *Lepidochelys kempii*, and green sea turtles *Chelonia mydas*

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ABSTRACT: Investigating animal gut microbiomes can lead to a better understanding of their foraging preferences and their overall health. In this study, the fecal and cloacal microbiomes of 4 cold-stunned, frozen loggerhead *Caretta caretta,* 9 Kemp's ridley *Lepidochelys kempii*, and 5 green sea turtles *Chelonia mydas* that stranded on beaches in Massachusetts, USA, were surveyed. Cloacal swabs and *in situ* fecal samples were collected from each turtle. From the extracted DNA, the hypervariable V1–V3 regions of the 16S rRNA gene were amplified with PCR, then sequenced using next generation Illumina MiSeq technology. Fecal and cloacal microbiomes were primarily composed of the phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*. Microbial communities varied significantly based on location of the gut sampled. Cloacal samples were largely dominated by *Proteobacteria*, while fecal samples appeared to have a greater distribution of taxa and higher alpha diversity. Green turtles had a higher abundance of *Firmicutes* and *Bacteroidetes* than Kemp's ridley and loggerhead turtles, but a lower abundance of *Proteobacteria*. The information gained from this study contributes to knowledge of cold-stunned sea turtle gut microbiomes and may eventually be applied to rehabilitation efforts.

KEY WORDS: Sea turtle · Gut microbiome · Cold-stunned · *Caretta caretta* · *Lepidochelys kempii* · *Chelonia mydas*

1. INTRODUCTION

Gut microbiota play a vital role in supporting the host's health and nutrition, including providing vitamins, contributing to immune system development, and regulating homeostasis (Flint et al. 2012, Hooper et al. 2012, LeBlanc et al. 2013, Sommer & Bäckhed 2013). Gut microbes can expand the host's metabolic potential by utilizing otherwise indigestible complex food particles such as polysaccharides (Flint et al. 2012, Sommer & Bäckhed 2013). Commensal bacteria bolster host immunity by attacking invading pathogens, stimulating host antimicrobial responses, and directing the differentiation and growth of immune cells (Ivanov & Honda 2012). The composition of gut microbiota is influenced by factors such as diet, physiology, lifestyle, antibiotic use, and habitat (Ravussin et al. 2012, Sullam et al. 2012, Sommer & Bäckhed 2013). If the composition of these microbial communities is disturbed, immune system function can become dysregulated, which leads to harmful inflammation and prolonged dysbiosis (an imbalance of the microbiota) (Round & Mazmanian 2009, Dickson et al. 2014). Dysbiosis is associated with several health disorders, such as colitis, liver disease, inflammatory and autoimmune diseases, and cancer (Costa et al. 2012, Tilg et al. 2016, Duvallet et al. 2017).

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Insight into the core microbiome (or set of microbes common across multiple microbial assemblages) of an animal is useful because any microbes that are persistent between individuals are likely to be beneficial to the host (Shade & Handelsman 2012, Apprill et al. 2017). Establishing the core microbiome for the gastrointestinal (GI) tract of sea turtles will lead to a better understanding of their health states and overall gut health, which is of particular importance as GI disorders are frequently diagnosed among stranded sea turtles (Flint et al. 2010, Ahasan et al. 2017). Knowledge of the gut microbiome can be applied to the management of sea turtle rehabilitation efforts, potentially impacting feeding and treatment protocols during hospitalization (Ahasan et al. 2018, Bloodgood et al. 2020, McNally et al. 2021b). With significant advances in biotechnology, sampling and analyzing gut microbiomes may become a standard protocol for monitoring the health status of captive animals, similar to blood chemistry analyses (Song et al. 2018, Franco-Duarte et al. 2019). Any improvements to the conservation and care of these animals would be meaningful, as these animals face significant threats from many anthropogenic factors, such as bycatch and habitat destruction, and are regularly kept in rehabilitation facilities after stranding events (Seminoff 2004, Innis et al 2019).

The International Union for the Conservation of Nature (IUCN) lists the loggerhead turtle *Caretta caretta* as Vulnerable, the Kemp's ridley turtle *Lepidochelys kempii* as Critically Endangered, and the green turtle *Chelonia mydas* as Endangered (Seminoff 2004, Casale & Tucker 2017, Wibbels & Bevan 2019). These turtles are susceptible to 'cold-stunning', a condition characterized by severe hypothermia that occurs when sea surface temperatures drop below 10°C, which happens annually in temperate waters. Juvenile turtles seasonally migrate and forage in northern habitats during the summer, but if they do not migrate south early enough, the onset of cold waters can induce cold-stunning, e.g. such as in Cape Cod Bay, Massachusetts, USA (Still et al. 2005, Innis et al. 2009), causing some turtles to stop swimming and be washed ashore by tidal activity (Wyneken et al. 2006, McNally et al. 2021b). Between 25 and 50% of cold-stunned turtles are dead by the time they are stranded (K. M. Dourdeville & R. L. Prescott unpubl. data). Moribund turtles frequently present with medical disorders such as dehydration, metabolic disturbances, and pathological conditions of the digestive, neurologic, and respiratory systems (Innis et al. 2009, Stockman et al. 2013, Turner et al. 2021). Unfortunately, the number of cold-stunned turtles that occur in Cape Cod Bay and other locations has been increasing over the past several decades and is predicted to continue to rise, due at least in part to climate change and increased Kemp's ridley and green turtle nesting attributed to conservation efforts (Griffin et al. 2019).

Only recently has the gut microbiome of sea turtles been examined, with the majority of studies to date focusing on green sea turtles (Ahasan et al. 2017, 2018, 2020, Price et al. 2017, Campos et al. 2018, Bloodgood et al. 2020, McDermid et al. 2020, Scheelings et al. 2020a). The bacterial composition of their gut microbiomes was influenced by factors such as diet, health status, and transitions between life stages and environments (Ahasan et al. 2017, 2018, Price et al. 2017, Campos et al. 2018). Few studies have explored loggerhead and Kemp's ridley turtle gut microbiomes (Abdelrhman et al. 2016, Arizza et al. 2019, Biagi et al. 2019, Samuelson et al. 2020, Scheelings et al. 2020a,b, McNally et al. 2021a,b). Interestingly, Biagi et al. (2019) found that hospitalization did not significantly alter the taxonomic composition or diversity of gut microbiota in loggerhead turtles, but Samuelson et al. (2020) found the opposite for Kemp's ridley turtles. Many of these studies have been limited by factors such as sample size, collection methods, sampling location, and number of species examined, all of which impact microbiome composition findings. These discrepancies lead to difficulties in drawing comparisons between studies. However, in this relatively unstudied field, obtaining preliminary information is important for future research.

In the present study, the fecal and cloacal bacterial communities of cold-stunned turtles from 3 species were examined. The objective was to contribute to the small but growing field of sea turtle microbiome data, offering unique comparisons between species and gut location while controlling for local environmental factors. It was hypothesized that there would be differences in bacterial composition between the species as well as location of the gut sampled.

2. MATERIALS AND METHODS

2.1. Sample collection

Each late fall/early winter, Mass Audubon's Wellfleet Bay Wildlife Sanctuary (WBWS) conducts an extensive rescue and recovery effort for cold-stunned sea turtles along the beaches of Cape Cod Bay, Massachusetts, USA. When turtles wash ashore dead, the carcasses are frozen for later necropsy. The turtles

analyzed for this paper were collected by WBWS during the 2019 cold-stun season, under the US Fish and Wildlife Service permit to NOAA Fisheries, TE01150C-1. Necropsies were conducted by WBWS. As they are conducted on a very limited budget under time and resource constraints, only 18 of the least-decomposed turtles out of the hundreds of individuals that were cold-stunned during the 2019 season were selected for sampling. Selection was based on an evaluation of decomposition by experienced WBWS staff, which includes an assessment of external appearance and presence or lack of odor. Of the 18 turtles, 4 were subadult loggerheads, 9 were juvenile Kemp's ridley, and 5 were juvenile green turtles.

Before their scheduled necropsies, loggerhead turtles were defrosted for 2 wk and the smaller Kemp's ridley and green turtles for 1 wk in a 4°C refrigerator. From each turtle's digestive system, 3 types of samples were collected in duplicate for a total of 6 samples per turtle. A sterile cotton swab was inserted into the cloaca and gently rolled along the luminal surface, collecting mucosal epithelia cells and small amounts of luminal contents. A second sterile swab was rolled on the luminal surface in the duodenum, just below the stomach and small intestine junction. Approximately 1 ml of fecal matter was collected by palpating the large intestine for feces and, if present, a small incision was made in the intestinal wall so that the feces could be removed and placed directly in a sterile vial. If no obvious large pieces of fecal matter were felt during palpations, the full length of the large intestine was sliced open and residual feces were collected using sterile equipment. All samples were stored in 1 ml of RNA*later*™ (Invitrogen). One set of the duplicate samples was used for DNA extraction and the other was archived at −80°C for later studies.

2.2. DNA extraction

DNA from cloacal and intestinal swab samples was extracted using DNeasy® PowerSoil® DNA Isolation Kit (Qiagen), with slight modifications to the manufacturer's protocol. Alterations included (1) incubating swab tips in the PowerBead solution and lysing agent (Solution C1) at 65°C for 15 min before vortexing, and (2) using 12.5 µl of the elution agent (Solution C6) and allowing it to remain on the filter membrane for 10 min, then repeating this step for a total elution volume of 25 µl. DNA from fecal samples was extracted using GeneMATRIX™ Tissue DNA Purification Kit (EURx), following the liquid tissues protocol with slight modifications. Alterations included (1) a 30 min incubation with the lysing agent (Sol T) at 70°C, with vortexing every 10 min, and (2) an elution volume of 25 µl instead of the recommended 50 to 150 µl. The 2 extraction methods were chosen based on preliminary testing of archived sea turtle feces. The DNeasy® PowerSoil® DNA Isolation Kit yielded larger quantities of higher quality DNA when extracting from swabs, while the GeneMATRIX™ Tissue DNA Purification Kit was more effective for the fecal matter. Once extracted, DNA was analyzed for quality and quantity using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific). All extracted DNA was stored at −20°C for short-term uses or at −80°C for long-term storage.

2.3. PCR amplification and sequencing

PCR was used to amplify the hypervariable bacterial V1−V3 regions of the 16S rRNA genes following the protocol from Ahasan et al. (2017). In brief, a 50 µl PCR reaction was carried out with 25 µl AmpliTaq Gold 360 mastermix (Life Technologies), using 5 µl of template DNA, and 0.2 µM of each of the following primers: 27F 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 519R 5′-GWA TTA CCG CGG CKG CTG-3′ with adapter overhang (Klindworth et al. 2013). Amplification was carried out using a T100™ Thermal Cycler (BioRad) starting with an initial denaturation at 95°C for 7 min, followed by 29 cycles at 94°C for 45 s, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min (Ahasan et al. 2017). Archived turtle feces from previous years were used as a positive control, while sterile water was used as a negative control. Using gel electrophoresis, DNA amplification was not detected in the small intestinal epithelial swab samples. DNA from the duplicate intestinal swab samples was also extracted; these samples similarly did not show DNA amplification, so were discarded. The PCR products resulting from the remaining sample types, cloacal swabs and fecal matter were sent to the Rhode Island Genomics and Sequencing Center for library preparation using the Illumina MiSeq™ and a 600 cycle Reagent Kit v3.

2.4. Data analysis

De-multiplexed paired-end reads were processed and filtered using Quantitative Insights into Microbial Ecology (QIIME version 2.0; Bolyen et al. 2019). The DADA2 pipeline (version 1.16) was used for removing primer sequences, quality trimming, and merging

paired-end reads (Callahan et al. 2016). Primer sequences, 20 bp from the forward and 18 bp from the reverse, were removed along with reads containing a quality score of <20. The reads were further quality filtered to remove sequences with a frequency per feature count of 45 or less, to minimize the inclusion of sequencing errors in downstream analysis. After initial processing, the reads were grouped into operational taxonomic units (OTUs). A representative sequence from each OTU was used to assign taxonomy using a SILVA 132 classifier trained for the bacterial 16s rRNA V1–V3 region based on a 97% similarity threshold (Quast et al. 2013). Rarefaction analysis was carried out by plotting the number of observed OTUs versus the total number of filtered reads for each sample. The samples were rarefied to an even depth of 30 000 for alpha and beta diversity analysis. Alpha diversity was measured using the Shannon-Wiener and Faith's phylogenetic diversity indices to compare differences between turtle species. Since the data were not normally distributed, as determined by visual inspection of Quantile-Quantile plots (R version 4.0.3, qqnorm function), pairwise Wilcoxon rank-sum tests with Holm p-values adjusted for multiple comparisons were used to examine differences in alpha diversity metrics between the species across each sample type. A multivariate approach was used to compare OTU abundance across sample type groups as well as species within those groups. This was carried out using an analysis of similarity (ANOSIM). Beta diversity was visualized using a non-metric multidimensional scaling (NMDS) plot of the Bray-Curtis dissimilarity metric with Adonis testing to compare microbial communities at the phylum level between cloacal swabs and fecal matter. Statistical analyses and visualizations were generated using R.

3. RESULTS

3.1. Turtle information

Mean (±SD) maximum straight carapace length (SCL) was 46.6 ± 8.7 cm for the loggerhead turtles, 25.0 ± 3.3 cm for the Kemp's ridley turtles, and 27.8 ± 1.3 cm for the green turtles (Table 1). During necropsy, over half of the Kemp's ridley turtles presented with parasitic cysts, and most turtles had sunken tissues around their eyes due to dehydration. However, some loggerhead turtles had fat deposits present, indicating fair nutrition despite recent cold-stunning. At necropsy, many of the turtles had fecal matter present in the large intestine, and some had recent meals in their stomachs. In general, the majority of these turtles were healthy before cold-stunning, but were very sick by the time they washed ashore.

Table 1. Demographic information for the 3 turtle species sampled. SCL_{nt}: straight carapace length notch to tip; CCL_{nt}: curved carapace length notch to tip. Values are mean ± SD

Species	Sample size	SCL_{nt} (cm)	CCL_{nt} (cm)	Weight (kg)
Chelonia mydas	5	27.8 ± 1.3	28.8 ± 1.4	2.6 ± 0.5
Lepidochelys kempii	9	25.0 ± 3.3	25.9 ± 3.6	2.4 ± 0.8
Caretta caretta	4	46.6 ± 8.7	50.0 ± 9.5	16.6 ± 9.3

3.2. Sequencing results and data analysis

Illumina MiSeq™ sequencing of the V1–V3 region of the 16s rRNA gene generated 13 909 858 raw reads from 18 fecal and 18 cloacal samples (as previously stated, small intestinal samples were not sent for sequencing because no DNA amplification was detected in them). Sequences were deposited to NCBI's Sequence Read Archive database (Accession #PRJNA813840). A total of 2 731 524 high quality, merged reads remained after filtering and removal of chimeras. Each sample contained an average of 75 876 sequences, which ranged in length from 280 to 500 bp, with the mean length being 477 bp. These sequences were grouped into 412 unique OTUs that were classified to the species level, spanning 14 phyla, 24 classes, and 112 families. OTUs that could not be assigned were labeled as 'Unclassified'. Cloacal and fecal samples had significantly different OTU abundance at the phylum level ($p = 0.0001$, $R = 0.5274$) (Fig. 1). Within each sample type, there was no significant difference for OTU abundance between turtle species (cloacal: $p = 0.6485$, $R = 0.6504$; fecal: $p = 0.2637$, $R = 0.04532$).

3.3. Alpha diversity

Loggerhead turtles had the lowest fecal and cloacal alpha diversity compared to the other 2 species, and cloacal samples had lower diversity values than fecal samples in both Shannon index (Fig. 2) and Faith's phylogenetic diversity (Fig. 3). Their

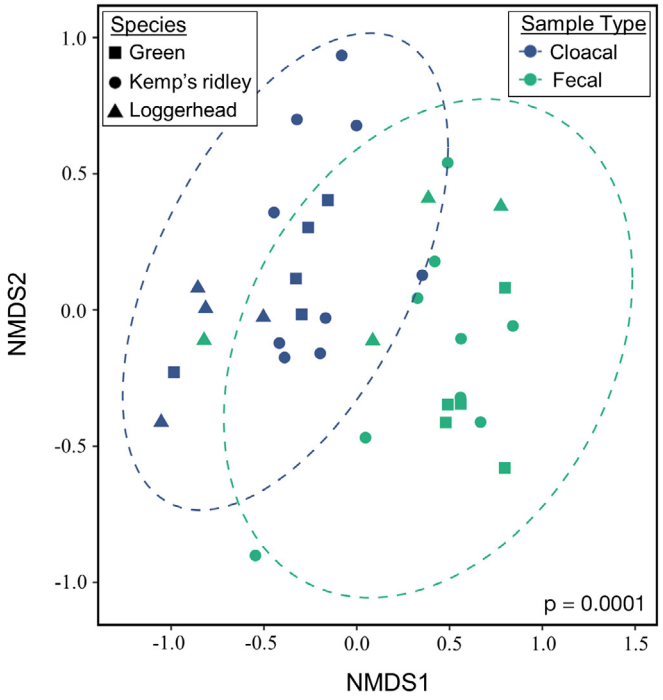

Fig. 1. Non-metric multidimensional scaling (NMDS) analysis of the dissimilarity between bacterial phyla in cloacal versus fecal samples. Each symbol represents 1 turtle, with color representing sample type and shape representing species

average (±SD) Shannon index values were 2.34 ± 0.45 for cloacal samples and 3.98 ± 1.15 for fecal samples, while Faith's index values were 8.15 ± 1.82 for cloacal samples and 12.39 ± 3.99 for fecal samples. Loggerhead cloacal samples had significantly lower diversity compared to Kemp's ridley ($p = 0.0084$) and green ($p = 0.0317$) turtles based on Shannon's index. By Faith's index, loggerhead cloacal diversity was significantly lower than Kemp's ridley turtles ($p = 0.0084$), and while not significant ($p = 0.13$), still lower than green turtles. Loggerhead fecal diversity was not significantly lower than Kemp's ridley turtles by Shannon's index ($p = 0.33$) and Faith's index ($p = 0.15$), or green turtles, by Shannon's index ($p = 0.19$) and Faith's index ($p = 0.56$).

Kemp's ridley turtles had the highest cloacal alpha diversity in both metrics, and the highest fecal diversity by Faith's index (Figs. 2 & 3). Cloacal samples from Kemp's ridley turtles had lower diversity values than fecal samples for both metrics. Shannon index values averaged 4.39 ± 0.67 for cloacal samples and 4.45 ± 1.06 for fecal samples, while Faith's index values were 19.15 ± 5.25 for cloacal samples and 22.06 ± 10.59 for fecal samples. For Kemp's ridley turtles, diversity was not significantly different from green turtles in their cloacal microbiomes, by Shannon's index ($p = 0.44$) and Faith's index ($p = 0.61$), or their fecal microbiomes, by Shannon's index ($p = 0.29$) and Faith's index ($p = 0.15$).

Green turtles had relatively high alpha diversity compared to loggerheads, but tended to be lower than Kemp's ridley turtles (Figs. 2 & 3). Cloacal samples for green turtles had lower diversity values than fecal samples by the Shannon index, but higher values by Faith's index. Shannon index values were 3.91 ± 0.82 for cloacal samples and 5.45 ± 0.72 for fecal samples, while Faith's index values were 16.56 ± 5.24 for cloacal samples, and 14.51 ± 2.42 for fecal samples. The fecal microbiomes of green turtles did have the highest Shannon index values, though these differences were not significant compared to the other species.

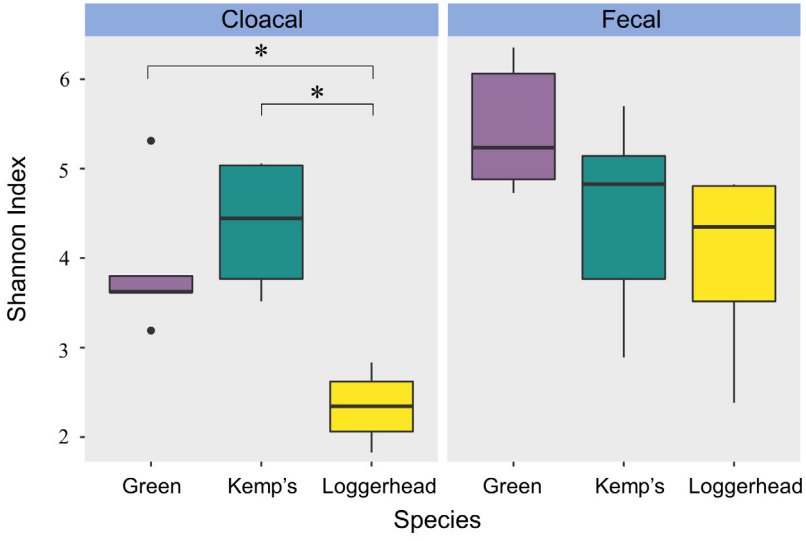

Fig. 2. Shannon diversity of bacterial phyla in cloacal versus fecal samples. Asterisk indicates significant difference based on pairwise Wilcoxon rank-sum test. Boxes represent all data between the lower first to the upper third quartile, with a line to represent median. Whiskers show minimum and maximum values, with dots representing outliers

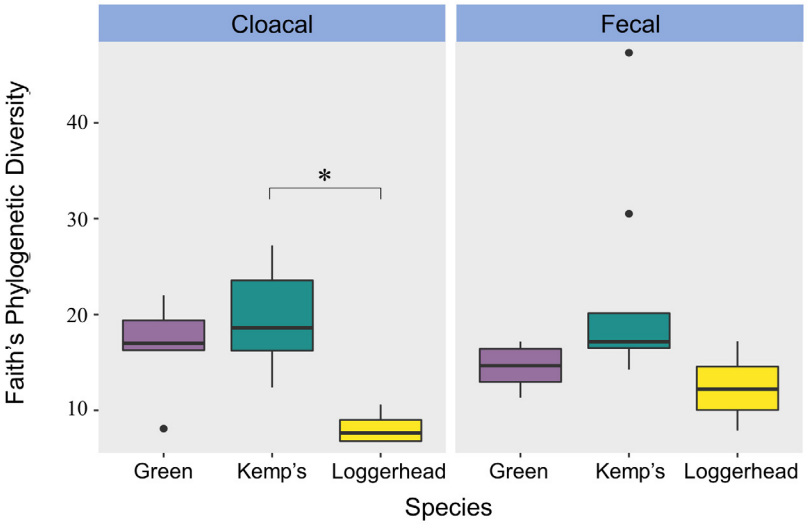

Fig. 3. Faith's phylogenetic diversity of bacterial phyla in cloacal versus fecal samples. Asterisk indicates significant difference based on pairwise Wilcoxon rank-sum test. Boxes represent all data between the lower first to the upper third quartile, with a line to represent median. Whiskers show minimum and maximum values, with dots representing outliers

3.4. Loggerhead turtle microbiome

The fecal microbiome for loggerheads was dominated by the bacterial phylum *Proteobacteria*, which had a mean abundance of 47.22% (Fig. 4). Also identified were the phyla *Firmicutes* (26.19%), *Fusobacteria* (16.45%), and *Bacteroidetes* (9.16%). In the cloacal microbiome, *Proteobacteria* composed the vast majority (97.03%) of bacteria found (Fig. 4). However, the cloacal microbiome also included organisms from *Fusobacteria* (2.21%), *Epsilonbacteraeota* (0.44%), *Firmicutes* (0.17%), and *Bacteroidetes* (0.11%). At the family level, the fecal microbiome appeared more evenly dispersed, including *Vibrionaceae* (29.19%) and *Enterobacteriaceae* (15.23%) (phylum *Proteobacteria*), *Fusobacteriaceae* (16.45%) (phylum *Fu-*

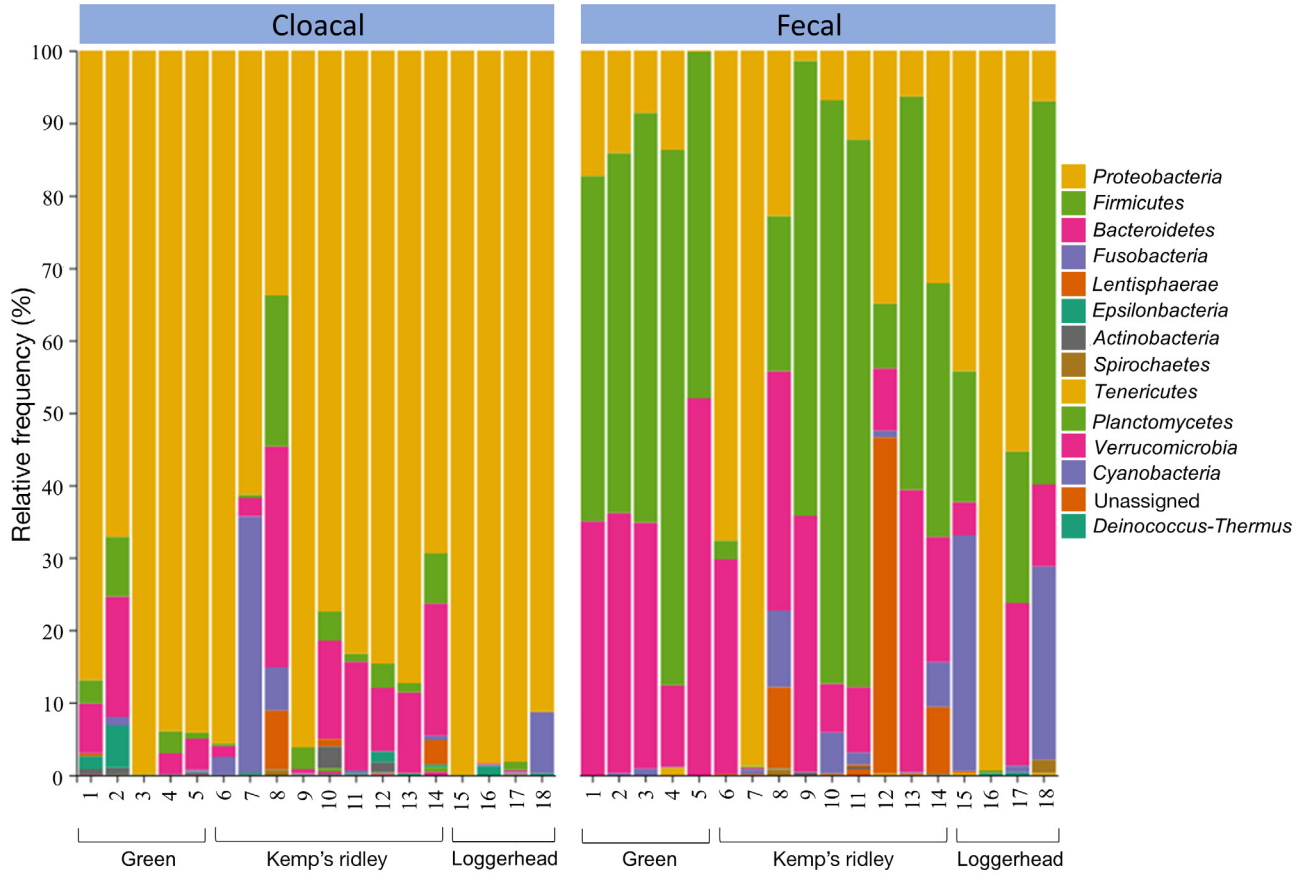

Fig. 4. Composition of turtle gut microbiomes. Relative abundances of bacterial phyla across each turtle sample, by species and sample type. Each number on the *x*-axis corresponds to an individual turtle

sobacteria), *Peptostreptococcaceae* (15.78%) (phylum *Firmicutes*), and *Tannerellaceae* (4.79%) (phylum *Bacteroidetes*) (Fig. 5). Prevalent taxonomic families found in the cloacal microbiome included *Enterobacteriaceae* (63.93%), *Vibrionaceae* (17.27%), and *Shewanellaceae* (7.61%) (phylum *Proteobacteria*) (Fig. 5).

3.5. Kemp's ridley turtle microbiome

The fecal microbiome of Kemp's ridley turtles consisted of the phyla *Proteobacteria* (35.33%), *Firmicutes* (35.13%), *Bacteroidetes* (19.5%), *Lentisphaerae* (6.83%), and *Fusobacteria* (2.89%) (Fig. 4). The cloacal microbiome had a different composition, being dominated by *Proteobacteria* (77.65%), *Bacteroidetes* (10.52%), *Fusobacteria* (5.24%), and *Firmicutes* (4.22%) (Fig. 4). At the family level, the fecal microbiome was composed of *Vibrionaceae* (29.92%) (phylum *Proteobacteria*), *Peptostreptococcaceae* (17.18%) (phylum *Firmicutes*), and *Tannerellaceae* (17.74%) and *Rikenellaceae* (6.55%) (phylum *Bacteroidetes*) (Fig. 5). The most prevalent taxonomic families present in the cloacal microbiome were *Enterobacteriaceae* (33.27%), *Vibrionaceae* (13.80%), *Moraxellaceae* (7.36%), *Shewanellaceae* (6.74%), and *Rhodobacteraceae* (3.89%) (phylum *Proteobacteria*), and *Flavobacteriaceae* (4.48%) (phylum *Bacteroidetes*) (Fig. 5).

3.6. Green turtle microbiome

The fecal microbiome for green turtles was dominated by the phyla *Firmicutes* (55.21%), *Bacteroidetes* (33.65%), and *Proteobacteria* (10.68%) (Fig. 4). The cloacal microbiome appeared less evenly dispersed, being largely dominated by *Proteobacteria* (90.86%), along with *Bacteroidetes* (4.87%), and *Firmicutes* (2.68%) (Fig. 4). At the family level, the fecal microbiome consisted of *Lachnospiraceae* (24.50%), *Ruminococcaceae* (10.83%), and *Clostridiaceae* (3.99%) (phylum *Firmicutes*), and *Tannerellaceae* (17.74%) and *Bacteroidaceae* (10.45%) (phylum *Bacteroidetes*) (Fig. 5). Prevalent taxonomic families present in the cloacal microbiome included *Enterobacteriaceae* (48.39%), *Moraxellaceae* (19.60%), and *Shewanellaceae* (10.36%) (phylum *Proteobacteria*) (Fig. 5).

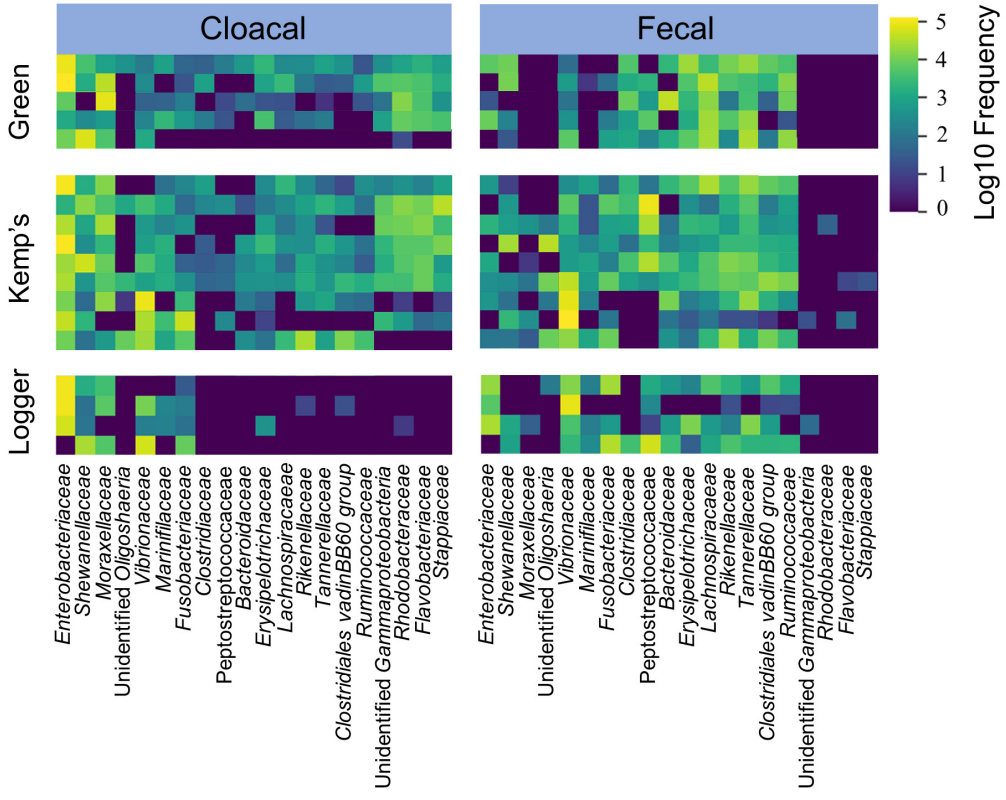

Fig. 5. Relative frequencies of the top 20 most abundant bacterial families across each turtle sample. Turtles are grouped by species and sample type

4. DISCUSSION

4.1. Use of tissues from frozen cold-stunned turtles

All turtles were collected from the shores of Cape Cod Bay, Massachusetts, USA, which may control for geographical influences on their gut microbiomes. The turtles in this study were cold-stunned, a condition associated with altered physiology and predisposition to microbial infections. This may allow opportunistic bacteria to take advantage of their diminished immune system and colonize their gut (Li et al. 2015, Ahasan et al. 2017). While compromised, cooling bay waters slowed their metabolism and led to changes or cessation of foraging behaviors, resulting in diminished nutrients ingested (Ahasan et al. 2017). Reduced foraging, along with the possible colonization and overgrowth of opportunistic bacteria, has the potential to heavily influence the composition and relative abundances of gut microbiota in unique and stochastic ways, and therefore these cold-stunned turtles may not be representative of healthy turtles of the same size and species (Zaneveld et al. 2017). In order to fully grasp the impact of gut microbiome on the health of these animals, it is important to have a detailed understanding of a 'normal' gut microbiome, along with possible deviations (Ahasan et al. 2017). Examining the gut microbiomes of unhealthy animals provides insight into disease states, which is necessary for identifying how diseases alter a healthy microbiome and could lead to improved diagnostics or therapies (Apprill et al. 2017, Moffatt & Cookson 2017, Bloodgood et al. 2020). The turtles in this study were sampled after their death but showed the lowest levels of decomposition out of all the dead cold-stunned turtles collected in the 2019 season. Additionally, the rapid freezing and slow thawing process strongly limits the reproduction and overgrowth of any bacteria, either previously residing in the turtles or newly introduced following stranding. Therefore, the microbiome composition of these turtles is not likely to be significantly altered post-mortem, and these dead turtles can still provide insight into the cold-stunned state of live turtles. If certain taxa can be identified as indicators of cold-stunning or diseases such as pneumonia, they might be able to be targeted during treatment, improving the recovery prospects of the hundreds or thousands of cold-stunned turtles that strand in Cape Cod Bay each year (Griffin et al. 2019).

However, the use of dead turtles for sampling has limitations, as the swabs collected from the small intestine showed no DNA amplification via PCR. This is possibly due to the rapid decomposition of the small intestine immediately following death, which contains high quantities of digestive enzymes. Future studies on dead cold-stunned turtles could benefit by sampling turtles that died in rehabilitation and were immediately frozen rather than ones collected from the beach after spending an unknown amount of time decomposing.

4.2. Fecal microbiomes

Collecting fecal and cloacal samples from 3 species offered unique comparisons between species and gastrointestinal (GI) tract locations. Fecal matter and cloacal swabs are often used in the place of *in situ* GI tract samples in sea turtle microbiome investigations, as *in situ* GI samples are practically impossible to obtain from live turtles (Price et al. 2017, Biagi et al. 2019). Prior to this study, *in situ* samples of the GI tract collected from dead turtles have been examined only minimally, in green turtles (Ahasan et al. 2020, McDermid et al. 2020). Ahasan et al. (2020) and McDermid et al. (2020) both identified *Firmicutes* and *Bacteroidetes* as dominant phyla throughout the GI tract of green turtles. In this study, *Firmicutes* and *Bacteroidetes* were the 2 most prevalent phyla in the fecal microbiome of green turtles, whereas cloacal samples were almost entirely composed of *Proteobacteria*. Additionally, the fecal microbiomes shared a similar composition, at the family level, to each of the individual GI sections examined by McDermid et al. (2020), while cloacal microbiomes did not. In all species, there was a significant difference in the relative abundances of bacterial phyla between fecal and cloacal microbiomes, and fecal microbiomes tended to have higher alpha diversity than cloacal ones. These results suggest that fecal microbiomes may provide more information about the GI tract compared to cloacal microbiomes. Although McDermid et al. (2020) argue that fecal samples may be unreliable compared to *in situ* samples of the GI tract, they appear to be a closer representation than cloacal microbiomes. This could be due in part to fecal microbiomes being less influenced by external ambient bacteria, in contrast to cloacal microbiomes which are directly exposed to the environment and aerobic conditions (Price et al. 2017, Biagi et al. 2019). However, these results should be interpreted with caution, as the fecal samples from the present study were collected from within the large intestine, and it is possible that the microbiome could be

altered as the fecal matter eventually becomes exposed to aerobic conditions in the cloaca. As this is the first study to directly compare fecal matter to cloacal samples from loggerhead and Kemp's ridley turtles, more research is needed to understand the differences between these sample types and their usefulness as health, diet, or life stage indicators.

The major phyla found in the fecal microbiome of loggerhead turtles of this study, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Fusobacteria*, have previously been identified as dominant in the fecal microbiomes of stranded loggerheads from coastal regions of Italy, though relative abundances varied (Abdelrhman et al. 2016, Arizza et al. 2019, Biagi et al. 2019). These findings suggest that these phyla, which are also dominant in the fecal microbiomes of some other carnivorous marine mammals and reptiles, are important contributors to the health of loggerhead turtles (Keenan et al. 2013, Nelson et al. 2013, Numberger et al. 2016). In the present study, loggerhead turtles had the highest levels of *Proteobacteria*, followed by Kemp's ridley and then green turtles, a trend which may influenced in part by the dietary preferences of these animals. Green turtles being fed animal protein during rehabilitation showed higher levels of *Proteobacteria* compared to wild-caught, herbivorous turtles or those fed diets lower in protein (Campos et al. 2018, Bloodgood et al. 2020). Loggerhead and Kemp's ridley turtles are primarily carnivorous, which could drive a higher prevalence of *Proteobacteria* compared to the more omnivorous green turtle (Bjorndal 1997, Howell & Shaver 2021). The same trend was noted for *Fusobacteria*, which is similarly associated with a carnivorous diet in marine mammals (Nelson et al. 2013). However, since these turtles are cold-stunned and have diminished or altered foraging behaviors, diet is likely to play less of a role in their microbiome composition. *Proteobacteria* was found at a higher prevalence in the loggerheads of this study compared to previous ones featuring stranded loggerheads (Abdelrhman et al. 2016, Arizza et al. 2019, Biagi et al. 2019). In mammals, elevated levels of *Proteobacteria* are an indicator of illness and dysbiosis known to worsen existing health conditions (Shin et al. 2015). Additionally, Scheelings et al. (2020a) found *Proteobacteria* to be the most abundant phylum in the distal colon of healthy, nesting loggerhead turtles undergoing a prolonged period of inappetence. Therefore, the heightened prevalence of *Proteobacteria* in this study could be due to a combination of the turtle's diminished health state and its lowered appetite and metabolism that occur as a result of cold-stunning (McNally et al. 2021b). Since all loggerhead fecal microbiome studies thus far have featured stranded turtles, additional research examining normal, healthy individuals is necessary in order to fully determine the roles that the dominant bacterial phyla play in the health and nutrition of these animals. Using the Shannon index, loggerhead turtles had significantly lower diversity than Kemp's ridley and green turtles in their cloacal microbiome, a result which contradicts Scheelings et al. (2020a). This difference between studies might be due to the smaller sample size, geographic location of the turtles sampled, and their diet or health status, all factors which impact microbiome composition (McNally et al. 2021b). The fecal microbiome of Kemp's ridley turtles has been examined by one other study, which sampled healthy turtles at the end of their rehabilitation period after incidental capture in the Gulf of Mexico (Samuelson et al. 2020). The present study identified the same major phyla, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Fusobacteria*, but also found high levels of *Lentisphaerae*, which Samuelson et al. (2020) only found in trace amounts. Our samples diverged from Samuelson et al. (2020) at the family level and had differences in relative abundance between the most prevalent phyla. This contrast might be explained by the differing health status, geographical location, and/or diet (natural versus captive-fed), which have been shown to impact sea turtle gut microbiomes (Price et al. 2017, Campos et al. 2018, Bean & Logan 2019, Ramirez et al. 2020, Samuelson et al. 2020, Scheelings et al. 2020b). However, *Bacteroidetes* and *Firmicutes*, which are common inhabitants in other carnivorous reptiles, were highly prevalent in both the present study and Samuelson et al. (2020), suggesting their importance to the gut function of Kemp's ridley turtles (Costello et al. 2010, Colston et al. 2015). Both phyla are commonly associated with herbivory and contain members well known for the digestion of complex carbohydrates into volatile short-chain fatty acids that are absorbed by the host (Hong et al. 2011, Thomas et al. 2011, Ahasan et al. 2018). In Kemp's ridley and loggerhead turtles, their role is uncertain. Those phyla may aid in the turtle's digestion of incidentally ingested food items, such as small amounts of seagrass, seaweed, or macroalgae (Arizza et al. 2019, Ramirez et al. 2020). However, marine *Bacteroidetes* also produce high levels of peptidases, suggesting their specialty in the degradation of proteins, which constitutes the majority of these carnivorous turtles' diets (Fernández-Gómez et al. 2013). Additionally, the *Firmicutes* in Kemp's ridley and loggerhead turtles was primarily composed of

the *Peptostreptococcaceae* family, which contains members that play a role in metabolizing proteins found in meat (Koeth et al. 2013). Therefore, it is possible that the members of *Firmicutes* and *Bacteroidetes* found within loggerhead and Kemp's ridley turtles could aid in the digestion of animal proteins, along with small amounts of plant matter. More research is required in order to fully understand the roles that bacteria play in the health of these animals. For example, the presence of *Peptostreptococcaceae* may also be an indicator of dysbiosis, as that family has been associated with disease in marine mammals and was found in a higher abundance in stranded green turtles compared to healthy, wild-caught ones (Nielsen et al. 2013, Ahasan et al. 2017).

The fecal microbiome of green turtles has been investigated more thoroughly than that of other species. Bloodgood et al. (2020) examined the fecal microbiome of stranded green turtles rescued from Georgia, USA, and sampled throughout their hospitalization. At intake, they identified *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* to be the most prevalent phyla, similarly to the present study, though *Proteobacteria* prevalence decreased by the end of the turtle's treatment period (Bloodgood et al. 2020). *Firmicutes* and *Bacteroidetes* also comprised the majority of the fecal microbiomes of healthy, wild-caught green turtles from Brazil and the Gulf of Mexico, with *Proteobacteria* present but in lower amounts than were identified in this study (Price et al. 2017, Campos et al. 2018). Our results suggest that *Firmicutes* and *Bacteroidetes* are major members of the green sea turtle fecal microbiome, regardless of the health state of these animals. Green turtles had higher levels of *Firmicutes* and *Bacteroidetes* compared to Kemp's ridley and loggerhead turtles, which may be due to their largely herbivorous diet. Within *Firmicutes*, *Lachnospiraceae* and *Ruminococcaceae* were the most abundant families in green turtles. These families play critical roles in fundamental metabolic conversions within the GI tract and could allow green turtles to harvest more energy from primary diet items like seagrass, which contain complex carbohydrates such as cellulose, hemicellulose, and xylan (Flint et al. 2012, Price et al. 2017, Ahasan et al. 2018). *Proteobacteria* probably plays a lesser role in the overall health of green turtles, and its prevalence in this study could be due to the malnourished condition of the cold-stunned animals, as stranded green turtles have higher levels of *Proteobacteria* in their fecal microbiome than those being fed a captive diet during rehabilitation (Bloodgood et al. 2020).

4.3. Cloacal microbiomes

This is the first study to compare fecal and cloacal microbiomes for loggerhead and Kemp's ridley turtles. The cloacal microbiomes were significantly different from the fecal microbiomes in terms of taxonomic composition and tended to have lower alpha diversity. Aerobic conditions in the cloaca, along with external influences such as water, air, and the beach, could impact the composition of the microbiome, leading the cloaca to be dominated by *Proteobacteria* (Price et al. 2017). Loggerhead samples were almost entirely composed of *Proteobacteria*, with low amounts of *Fusobacteria*, while Kemp's ridley and green turtle samples contained higher levels of *Bacteroidetes* and *Firmicutes* but were still dominated by *Proteobacteria*. A high abundance of *Proteobacteria* (78 to 97%) in the cloacal microbiome of these 3 species is distinct compared to previous studies, so the fact that these turtles were cold-stunned and spent an unknown period of time decaying on the beach is an important consideration (Price et al. 2017, Scheelings et al. 2020b, McNally et al. 2021b). Cloacal samples had a higher prevalence of *Shewanellaceae*, the family that composes the dominant bacteria in spoiled fish meat and seafood, which could be a result of tissue decaying as the turtles died before washing ashore or laid on the beach for up to a few hours before being collected and frozen for this study (Zhuang et al. 2021). In addition to *Shewanellaceae*, cloacal samples had a high prevalence of other *Gammaproteobacteria* families, such as *Enterobacteriaceae*, *Vibrionaceae*, and *Moraxellaceae*, which are known to cause infections in sea turtles and a variety of other aquatic species, including marine and freshwater fish, crustaceans, and echinoderms (Raidal et al. 1998, Work et al. 2003, Austin & Zhang 2006, Paździor 2016). More research is required to determine if these families are normal inhabitants of the cloaca or are a result of cold-stunning. The lower diversity of cloacal microbiomes, along with the potential for environmental contamination, suggest that fecal microbiomes are more representative of the greater GI tract.

Cloacal microbiomes of live, rehabilitating cold-stunned Kemp's ridley turtles, also collected in Cape Cod and sampled at intake, have been examined by McNally et al. (2021b), who identified *Vibrionaceae*, *Arcobacteraceae*, *Shewanellaceae*, and *Rhodobacteraceae* as the most prevalent families. The present study found each of these families in comparable amounts, with the exception of *Arcobacteraceae*, which was only found in trace amounts. Additionally,

the present study identified high amounts of the *Proteobacteria* families *Enterobacteriaceae* and *Moraxellaceae*. The similarities between the present study and McNally et al. (2021b) suggest that dead cold-stunned turtles can serve as a reasonable proxy for live cold-stunned turtles. The differences between the 2 study groups might be explained by the years the turtles were sampled (2015 vs. 2019), or the smaller sample size of 9 turtles for the present study. Further research comparing dead cold-stunned turtles to live ones is necessary to determine if the microbiome composition undergoes significant changes post-mortem.

5. CONCLUSIONS

This study contributes to the limited but growing knowledge of sea turtle gut microbiomes. The data obtained here help establish preliminary data for dead cold-stunned sea turtle microbiomes. Despite not featuring live turtles in this study, these data help characterize the ways that healthy gut microbiomes can shift with declining health, which may prove useful for understanding disease states and redesigning treatment protocols. Although dead cold-stunned turtles were sampled, they still retained fecal and cloacal microbiomes that were similar to live unhealthy turtles, indicating that the composition of bacteria did not degrade substantially post-mortem. A significant difference in bacterial abundance between fecal and cloacal microbiomes was identified, and cloacal microbiomes were found to have lower diversity. These findings suggest that *in situ* fecal microbiomes might be a better representation of the overall diversity or composition of GI tract microbiome when compared to cloacal microbiomes. This may be important information for future microbiome sampling, especially if gut microbiome analysis becomes a standard protocol in health monitoring.

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