HEALTH PARAMETERS IN THE LEATHERBACK SEA TURTLE (*DERMOCHELYS CORIACEA*) AND THE IMPACT OF CLIMATE CHANGE ON THEM

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DEDICATION

I dedicate this work to my grandmother, Joyce Kuschke, who always encouraged me to be inquisitive and appreciate the outdoors.

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ABSTRACT

The leatherback sea turtle (*Dermochelys coriacea*) is the largest species of sea turtle, and the only extant genus of Dermochelyidae. Within the United States, all populations of leatherbacks are considered endangered. As such, great efforts are aimed at population conservation; however, many of these efforts are thwarted by a fundamental lack of baseline health knowledge of the species and how their health is impacted by climate change. In an effort to fill some of these knowledge gaps I established reference intervals for multiple blood analytes (packed cell volume, estimated total protein, and agarose gel electrophoresis) in leatherback neonates at emergence and at 3-4 weeks of age after captive-rearing. I also characterized the skin microbiota of the leatherback sea turtle at multiple life-stage classes. These data serve as an essential starting place for understanding health in leatherback sea turtles and a provide novel means to measure it. To assess how climate change is affecting leatherback health I studied the impact of rising incubation temperatures on these measures of health in neonates at emergence and again at 3-4 weeks. I found significant alterations in blood analytes (packed cell volume, estimated total protein, pre-albumin, alpha-1 globulins, and alpha-2 globulins) when compared between turtles that incubated in cooler versus hot nests at both emergence and 3-4 weeks of age. Additionally, I observed significant shifts in the skin microbiota between temperature groups at both time points. These data reveal that increasing incubation temperatures are altering neonatal physiology at emergence, and that significant differences can persist for up to 3-4 weeks. Consequently, the impacts of increasing incubation temperatures have potential long-term negative impacts on neonatal health and thus survival. In the future, conservation efforts should aim to address negative impacts of climate change on health to mitigate the potential long-term impacts of increasing incubation temperatures on leatherback neonates.

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INTRODUCTION

Climate change is a growing threat to humans, animals, and plants around the world. The effects of climate change are broad and span a range of negative impacts on environments, economies, human, animal, and plant health, agriculture, biodiversity, and more (Anderson et al., 2004; Ebi et al., 2006; Chen et al., 2011; Eastburn et al., 2011; Calzadilla et al., 2013; Rinawati et al., 2013; Thornes, 2016; Tol, 2018; Simantiris, 2024). Many of these negative impacts, especially those on health, are linked to rising global temperatures (Root et al., 2003; Brierley and Kingsford, 2009; Garcia-Solache and Casadevall, 2010; Chen et al., 2011; Isler et al., 2023). Increasing environmental temperatures are shifting species distributions, altering interspecies competition, increasing physiologic stress, and leading to an increased prevalence of infectious disease processes (Anderson et al., 2004; Helmuth et al., 2005; Le Roux and McGeoch, 2008; Marcogliese, 2008; Sachan and Singh, 2010; Chen et al., 2011; One Health, 2019; Isler et al., 2023; Walberg, 2024). The negative impacts of rising temperatures are more exacerbated in animals like sea turtles that must overcome these threats during multiple stages of their life cycle (Hamann et al., 2010; Fuentes et al., 2012; NMFS and US FWS, 2020). Consequently, many sea turtle species currently face an increased risk of extinction partly due to the negative impacts of climate change (IUCN 2023).

Some of the current recommended and possible conservation strategies to support sea turtle populations include but are not limited to implementing legal protection for key environments, reducing bycatch, manipulating in situ incubation environments, ex situ incubation trials, and even head-starting programs (Cox et al., 2007; Fuentes et al., 2012; Jualaong et al., 2021; Kanghae et al., 2024). Though there are many proposed strategies for sea turtle conservation efforts, many questions remain regarding the appropriate application and impact of these activities (Hamann et al., 2010; Fuentes et al., 2011; Patrício el al., 2021; Simantiris, 2024). Currently, it seems that there are more questions than answers when it comes to addressing the conservation of sea turtle species. Hamann et al. (2010) identified some of the key areas in need of additional research. Within the priority research categories meta-questions included how do we identify a healthy turtle, how is climate change going to impact sea turtles, and how can we mitigate these effects (Hamann et al., 2010)? Accordingly, these data gaps urgently need filling and current research is expanding to accomplish this.

Unfortunately, knowledge gaps in some species may be more challenging to fill than others. The leatherback sea turtle (*Dermochelys coriacea*) is one such species. Currently, under the Endangered Species Act (ESA) of 1973, all populations are listed as endangered (NMFS and US FWS, 2020). The global International Union for Conservation of Nature (IUCN) has assigned the leatherback sea turtle a Red List Category of vulnerable, with declining numbers of mature individuals and many critically endangered subpopulations (IUCN 2023). The cause of the leatherback conservation status is multifactorial and thus population recovery depends on efforts fueled by a strong understanding of the species. However, current sea turtle research is dominated by studies on the Cheloniidae. This is likely due to the unique challenges posed by leatherbacks to researchers, including their oceanic-pelagic lifestyle (Jones et al., 2000; Jones et al., 2011). In addition to the difficulty of accessing this species, they are also incredibly difficult to maintain in research or rehabilitation settings (Jones et al., 2000; Jones et al.,

2011). Consequently, there is limited research performed outside of the two more easily accessed life-stage classes, nesting females and hatchlings. Although leatherbacks may be difficult to study, fundamental data gaps urgently need filling. As the sole extant genus of the family Dermochelyidae, the leatherback sea turtle is highly unique from other species of sea turtles. As such, inferences from other species are likely guesses rather than fact and may not translate to useful applications in conservation efforts.

In an effort to better understand what constitutes a "healthy" sea turtle, we must first understand that there are multiple methods to assess health. One of the more common ways to assess health is through the use of blood analytes. Blood analytes have been used to diagnose disease and assess the health of sea turtles in the wild and in rehabilitation settings (Stacy and Innis, 2017). However, when assessing the significance of a blood analyte, it is important to have reference intervals for comparison and interpretation of the obtained value (Horn and Pesce, 2003). Additionally, for the most accurate application of blood analytes in assessing health, reference intervals should be both species and age class-specific (Stacy and Innis, 2017; Stacy et al., 2018; Perrault et al., 2020). In Chapter II, I establish reference intervals (RIs) for blood analytes in leatherback neonates at two time points (emergence and at 3–4 weeks of age after captive-rearing). These baseline data can be used for health assessments of neonatal leatherbacks in the wild, research facilities, or head-starting programs. Reference intervals can also be used to observe changes in leatherback health over time in head-starting programs or in response to shifting environmental variables in the wild.

Another unique avenue to assess turtle health is through the microbiome. Recently, researchers have begun to understand the importance of the microorganisms living on and within humans and animals (Sommer and Backhed 2013; Selosse et al., 2014; Colston and Jackson 2016; Walke and Belden 2016; Simon et al., 2019). In Chapter I, I summarize the current literature available on the bacterial microbiota on and within sea turtles. This chapter serves as a reference work for others to understand the current information available and identifies areas where additional research is needed. Within this chapter, I identify an urgent need for further investigations into the skin microbiota on sea turtles. The skin microbiota is of great interest as it serves as a primary defense against the outside world and plays a significant role in the maintenance of host health (Harris et al., 2009; Selosse et al., 2014; Walke et al., 2015; Colston and Jackson 2016; Walke and Belden 2016; Jiménez and Sommer 2016; Simon et al., 2019). To begin filling this data gap, in Chapter III, I present skin microbiota data from leatherback sea turtles obtained through the use of next-generation sequencing. These baseline data on skin microbiota in leatherback sea turtles adds to the body of work that can now be used to identify a healthy leatherback sea turtle.

Within this work, I also begin to address how climate change is impacting leatherback sea turtle neonates. Recent studies have uncovered that increasing incubation temperatures are causing dehydration and possibly inflammation in sea turtle neonates (Fleming et al., 2020; Page-Karjian et al., 2022; Perrault et al., 2022). In Chapter II, I compare the values of different blood analytes between neonates incubated in hot versus cooler nests at emergence and again at 3–4 weeks of age. These data will not only expand our current knowledge and understanding of the impacts of increasing temperatures on

neonates at emergence, but also present data assessing the potential long-term impacts of increased incubation temperature on neonatal health. Additionally, in Chapter IV, I assess the impact of incubation temperature on the skin microbiota of neonatal leatherbacks at emergence and again at 3–4 weeks of age. Throughout the studies present here, I begin to fill some of the fundamental data gaps in current literature and identify how these data shift in response to increasing incubation temperatures.

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CHAPTER I WHAT LIVES ON AND IN THE SEA TURTLE? A LITERATURE REVIEW OF SEA TURTLE BACTERIAL MICROBIOTA

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Abstract

Within the United States, all populations of sea turtles are listed as threatened or endangered under the Endangered Species Act. Identifying methods of supporting health, preventing disease, and treating disease is essential for conservation and management strategies for all species. Over the last few decades, advances in technology and high throughput sequencing have allowed a proliferation of research into core microbiota and microbiomes in humans and animals. Such investigations have proven that microbiota on and within a host can influence physiology, immunity, and development. Accordingly, a comprehensive understanding of microbiota is essential for unearthing the complex relationships within a microbiome and how those interactions and relationships can be used to promote health and prevent or treat disease. The goal of this review was to summarize the current microbiota research available across all species of sea turtles and identify any emerging trends. Methodological differences made it challenging to draw conclusions across studies, but it is apparent that each anatomical location investigated has a unique core microbiota with some potential overlap. In the future, unifying methodology across microbiota studies will allow broader conclusions to be drawn across all anatomic locations and species of sea turtles. These conclusions will then allow clinicians and conservationists to apply the research results in the field. Additionally, future efforts should include a wider range of organisms including fungi, viruses, parasites, epibiota, and archaea to unveil essential relationships among and between the organisms and host for maintenance of a healthy microbiome.

Introduction

Most animals are home to a complex array of microbiota, which is defined as communities of microorganisms originating from multiple different kingdoms (Berg et al., 2020; Neu et al., 2021). Microbiota, in combination with their spectra of activity, make up what is known as a microbiome (Berg et al., 2020; Neu et al., 2021). This encompasses the microbial structures, metabolites, mobile genetic elements, and relic DNA (Ursell et al., 2012; Berg et al., 2020; Neu et al., 2021). Within a microbiome, there is a subset of microbes referred to as the core microbiota (Berg et al., 2020; Neu et al., 2021). Core microbiota are a subset of the microbiota that are shared across a species in similar habitats (Berg et al., 2020; Neu et al., 2021). A comprehensive understanding of core microbiota is essential for unearthing the complex relationships within a microbiome such as stability, plasticity, and individual purposes or functions (Ursell et al., 2012; Berg et al., 2020).

Through the last few decades, advances in technology and high throughput sequencing have allowed a proliferation of research into core microbiota and microbiomes (Ursell et al., 2012; Forde and O'Toole 2013; Gupta et al., 2019; Wensel et al., 2022). These advances have supported rapid and more affordable investigation into the microbiomes of human and nonhuman animals (Ursell et al., 2012; Forde and O'Toole 2013; Wensel et al., 2022). Interest in these studies is growing and stems from increasing understanding that microbiota can significantly impact host functions (Sommer and Backhed 2013; Selosse et al., 2014; Colston and Jackson 2016; Simon et al., 2019). Research thus far has revealed that microbiota may influence host physiology, immunity, and development (Selosse et al., 2014; Colston and Jackson 2016; Kho and Lal 2018; Simon et al., 2019). Significantly, the microbiota most investigated across all species include those of the gastrointestinal tract and skin.

The microbiota of the gastrointestinal tract aids in metabolic homeostasis, digestion, and provides key nutrients to the host (Shin et al., 2011; Sommer and Backhed 2013; Thursby and Juge 2017). More surprisingly, the gastrointestinal microbiota also plays an essential role in multiple host physiologic process (Shin et al., 2011; Sommer and Backhed 2013; Thursby and Juge 2017). Morphogenesis of the gastrointestinal tract as well as development of secondary gut associated lymphoid tissue within the intestines partially depends on postnatal microbial colonization (Sommer and Backhed 2013). The gastrointestinal microbiota can also modulate the differentiation of some subsets of immune cells and production of cytokines, chemokines, and other soluble immune mediators (Sommer and Backhed 2013, Selosse et al., 2014; Kho and Lal 2018). Additionally, intestinal vascularization, tissue regeneration, carcinogenesis, bone homeostasis, and behavior can all be influenced by the gastrointestinal microbiota (Stappenbeck et al., 2002; Shin et al., 2011; Reinhardt et al., 2012; Sommer and Backhed 2013).

Though somewhat less explored in animals, the skin microbiota is also a highly important network of microorganisms. The skin serves as a primary barrier and defense against the outside world (Harris et al., 2009; Sanford and Gallo 2013; Walke et al., 2015; Jiménez and Sommer 2016; Walke et al., 2016; Ross et al., 2019; Boxberger et al., 2021). Commensal microbiota of the skin aid in defense via production of inhibitory compounds or by competing for resources necessary for growth (Ross et al., 2019; Boxberger et al., 2021). Skin microbiota are believed to play a significant role in host health, host resistance, immune function, response to endogenous and exogenous stressors, and disease clearance (Harris et al., 2009; Sanford and Gallo 2013; Walke et al., 2015; Jiménez and Sommer 2016; Walke et al., 2016; Boxberger et al., 2021). A protective skin microbiota in amphibians has exhibited the ability to alter disease outcomes (Harris et al., 2009; Walke et al., 2016). Harris et al. demonstrated that the amphibian skin microbiome can decrease both morbidity and mortality associated with skin diseases caused by *Batrachochytrium dendrobatidis* (Bd) (Harris et al., 2009). Becker et al. identified significant differences in skin microbiota among Panamanian golden frogs that cleared Bd infections versus those that died, suggesting that skin microbiota play an important role in clearance of Bd (Becker et al., 2015).

Investigations into the microbiota of marine animals has also become an area of interest due to their constant contact with salt water (Apprill et al., 2011; Bik et al., 2016; Ruusso et al., 2018; Chiarello et al., 2017). Some of this work includes studies in marine turtles. There are seven extant species of sea turtles, most of which are imperiled. Within the United States, all populations are listed as threatened or endangered under the Endangered Species Act (United States. The Endangered Species Act as Amended by Public Law 97-304 (the Endangered Species Act Amendments of 1982)). They are also listed on the International Union for Conservation of Nature (IUCN) red list as vulnerable, endangered, or critically endangered (IUCN Red List). The only exception is the flatback turtle (*Natator depressus*), which is listed as data deficient. Marine turtles are often considered sentinels of the ocean due to their long-life spans and expansive migratory patterns (Aguirre and Tabor 2004). Accordingly, large research efforts are aimed at understanding the effects of climate change on the health, physiology, and disease processes of these sentinel animals to aid conservation efforts across the world (Aguirre and Tabor 2004). A small portion of this research has been aimed at gaining knowledge of the microbiota and microbiomes of sea turtles. The primary focus of microbiota research in sea turtles has been the microbiota contained within the gastrointestinal tract, with smaller portions of the research branching into the microbiota found in the cloaca, mouth, and nares (Table 1.1).

The principal goals of microbiota studies in sea turtles aim to gain knowledge and understanding to improve husbandry in rehabilitation settings, improve survival outcomes of eggs, and improve treatment and prevention of disease processes (Ahasan et al., 2018; Kittle et al., 2018; Bloodgood et al., 2020; Samuelson et al., 2020). In addition to these goals, a small subset of research has also been aimed at the identification of microorganisms that could serve as potential human pathogens, such as multidrug resistant bacteria (Alduina et al., 2020; Santoro et al., 2008; Zavala-Norzagaray et al., 2015; Blasi et al., 2020). The objective of this paper is to investigate the current microbiota research available from all species of sea turtles. Literature with a focus or clear relation to core microbiota of the sea turtle were the focus of this investigation. Using these essential studies, I summarize the microbiota found on and within the sea turtles, identify emerging trends, and highlight areas for future focus. I have organized the review into subsections covering different anatomical locations of the sea turtle. I begin with the gastrointestinal tract and subdivide this into sample types used. I then go into mucosal surfaces, the skin and the shell.

In 2021, the International committee on Systematics of Prokaryotes changed the names of multiple phyla to align with the International Code of Nomenclature of Prokaryotes. The research presented in the review was performed prior to this change and therefore I use the previously recognized names for phyla described. To maintain consistency among the literature being referenced, I use the previous phylum names (Table 1.2).

Gastrointestinal tract

Accurately sampling the gastrointestinal microbiota in living animals can be difficult. Due to the challenging nature of obtaining gastrointestinal samples, I have

categorized gastrointestinal microbiota research in several fashions. The first being whether the samples were obtained from live animals, or if the samples were taken following death, either natural or via euthanasia. While sampling post-mortem allows direct samples to be taken from different portions of the gastrointestinal tract, there are likely multiple factors that may have altered the microbiota prior to sampling such as illness, injury, anorexia, or dehydration (McNally et al., 2021a). Sampling from live sea turtles can also have biases depending on if the animal is wild caught, stranded, debilitated, or in a rehabilitation center (Ahasan et al., 2017; Samuelson et al., 2020). Additionally, samples used as a proxy (i.e., cloacal, and fecal samples) to estimate the gastrointestinal microbiota in living turtles can influence the results (McNally et al., 2021a). Across the literature cloacal swabs and fecal samples are often used to infer the microbiota population of the gastrointestinal tract, although there are several papers that sample the colon via deep swabs through the cloaca [Table 1]. The sample used and potential for contamination should always be taken into consideration when analyzing data in these studies.

Postmortem gastrointestinal samples

In 2018, Kittle et al. performed an investigation into the effect of the herbicide glyphosate on the microflora of green turtles (*Chelonia mydas*) in Hawaii (Kittle et al., 2018). Through traditional culture methods, they identified 4 taxa of bacteria from the gastrointestinal tract of 8 green turtles that required euthanasia. The taxa identified included *Proteus* sp., *Pantoea* sp., and *Shigella* sp.; all within the phylum Proteobacteria and the class Gammaproteobacteria. The fourth taxon identified was *Staphylococcus* sp. in the phylum Firmicutes. All 4 taxa proved to be sensitive to glyphosate in a dose dependent manner. It is important to note that the region of the gastrointestinal tract from which these taxa were isolated was not described and could range anywhere from the crop to the colon (Kittle et al., 2018).

Also using traditional culture techniques, in 2020, McDermid et al. investigated different sections of the gastrointestinal tract from 8 Hawaiian green turtles that required euthanasia after mortal injury or terminal illness (McDermid et al., 2020). Samples were collected from the crop, stomach, small intestine, cecum, and large intestine. Culture and biochemical reactions were used to identify 11 taxa within the phylum Proteobacteria and 2 isolates within the phylum Firmicutes. Among the sections of the gastrointestinal tract sampled, the small intestines had the most taxa isolated at 13, followed by the stomach and cecum each with 12 taxa identified. The large intestine had the fewest number of taxa isolated at only 6. In addition to the culture samples, 2 whole gastrointestinal tracts were used to sample the cecum, large intestines, and rectum. Using high through put sequencing, the most dominant phyla were identified as Firmicutes and Bacteroidetes. At the order level, Clostridiales and Bacteroidales predominated. Additionally, at the family level Clostridiaceae, Ruminococcaceae, Lachnospiraceae, Porphyromonadaceae, and Bacteroidaceae were most abundant (McDermid et al., 2020).

In 2020, Ahasan et al. performed a more comprehensive investigation of the mucosa-associated bacterial communities within the gastrointestinal tract of 4 stranded green turtles that died 1 to 4 days after being taken to a rehabilitation facility (Ahasan et

al., 2020). The oesophagus, stomach, small intestine, and large intestine were sampled in each turtle and high throughput sequencing was used to identify the microbiota present in each section. Across the entire gastrointestinal tract, the relative abundance (RA) of the dominant phyla were Firmicutes 57.8%, Proteobacteria 21.3%, Actinobacteria 6.4%, Bacteroidetes 3.6%, and Fusobacteria 2.4%. Firmicutes, Proteobacteria, and Actinobacteria were present in all regions of the gastrointestinal tract. In the small intestines, it was noted that there was a drastically lower abundance of Firmicutes and a significantly higher abundance of Proteobacteria. There were 30 dominant families identified that belonged to 11 different classes: Clostridia, Gammaproteobacteria, Bacilli, Actinobacteria, Bacteroidia, Alphaproteobacteria, Fusobacteria, Epsilonproteobacteria, Spirochaetes, Coriobacteria, and Erysipelotrichia. The nature of this study allowed comparisons between the sections of the gastrointestinal tract to be made. Some of the significant differences noted between sections included: (i) the absence of Actinobacteria in the large intestines and the higher abundance in the oesophageal samples, (ii) Peptostreptococcaceae was significantly more abundant in the stomach and large intestines, and (iii) Clostridia was one of the most abundant classes across all regions except in the small intestines which had a significantly lower abundance of that class. At the level of genera, a total of 459 taxa were identified across the whole intestinal tract but over 35.8% of the total sequences could not be identified. Of the operational taxonomical units (OTU) identified, 11 of them were shared across all gut regions. The phylum Firmicutes accounted for 8 of the 11 OTUs shared across the entire gastrointestinal tract. The other 3 OTUs fell within the phlya Proteobacteria, Bacteroidetes, and Fusobacteria. While this study provides a very in-depth look at the gastrointestinal microbiota of the green turtle, limitations of the study include the small sample size and the poor health status of the turtles (Ahasan et al., 2020).

Abdelrhman et al. (2016) found similar dominant microbiota in an investigation of loggerhead turtles (*Caretta caretta*) from the Tyrrhenian Sea east of Italy (Abdelrhman et al., 2016). A portion of the study used colorectal samples from 6 recently deceased loggerheads, from unspecified causes, and investigated the microbiota via high throughput sequencing. Like the findings in Ahasan et al. (2020), Firmicutes was the phylum with the highest percentage of occurrence. The percent of occurrence was reportedly higher in loggerheads at 87% (Abdelrhman et al., 2016) than in green turtles (Ahasan et al., 2020). Firmicutes was followed by the phyla Proteobacteria at 4.2% occurrence and Bacteroidetes at 3.4% occurrence. Furthermore, Abdelrhman et al. also found that the classes Clostridia and Bacilli dominated within Firmicutes with 43% and 42.5% occurrence respectively (Abdelrhman et al., 2016).

Across all postmortem samples, there appears to be a common thread at the phylum level. Firmicutes, Bacteroidetes, and Proteobacteria are consistently identified as predominant phyla, though found at differing abundances in each individual study. However, it is important to note that with all postmortem studies, the individual turtles sampled likely had several complex physiological abnormalities present, which are apt to alter the normal gastrointestinal microbiota. Therefore, these studies may represent the change one would expect to see in the microbiota of a sick, injured, or debilitated sea turtle. Additionally, the window between the time of death and the time of sampling

should also be considered when interpreting data from post-mortem samples. Extended post-mortem windows may allow for extensive amounts of bacterial overgrowth and may alter findings.

Fecal samples

Multiple studies have investigated the gut microbiome in sea turtles using next generation sequencing on fecal samples. Species sampled thus far include green turtles, Kemp's ridleys (*Lepidochelys kempii*) and loggerhead turtles. Campos et al. (2018) sampled 8 live captive and 11 live wild caught juvenile green turtles, while Samuelson et al. (2020) sampled 30 live incidentally captured juvenile Kemp's ridley turtles following rehabilitation for external injuries. Both Campos et al. and Samuelson et al. used next generation sequencing and identified Bacteroidetes as the most prominent phyla and Firmicutes as the second most abundant phyla in the fecal samples. A significant increase in the abundance of Proteobacteria was noted in association with prolonged captivity (Campos et al., 2018; Samuelson et al., 2020) and treatment with antibiotics (Samuelson et al., 2020). Both studies postulated that that increases in Proteobacteria could be an indication of dysbiosis in sea turtles (Campos et al., 2018; Samuelson et al., 2020). In direct contrast, Bloodgood et al. reported no significant differences between juvenile green turtles treated with and without antibiotics (Bloodgood et al., 2020). In 2020, Bloodgood et al. sampled feces from 17 individual sea turtles at three time points across rehabilitation (admission, mid-rehabilitation, and recovery) and performed next generation sequencing to identify bacteria present. Dominant phyla detected at recovery were Bacteroidetes (RA 38.4%), Firmicutes (RA 31.8%), Verrucomicrobia (RA 5.45%) and Proteobacteria (RA 1.8%) (Bloodgood et al., 2020), which aligns with the two dominant phyla identified by Campos et al. and Samuelson et al. In contrast, at admission, the most abundant phylum was Firmicutes (RA 55.0%), followed by Bacteroidetes (RA 11.4%) and Proteobacteria (RA 6.2%) (Bloodgood et al., 2020). If, as suggested by Campos et al. and Samuelson et al., an increase in Proteobacteria indicates dysbiosis, this would mean that the green turtles included in Bloodgood et al. had fecal dysbiosis at admission, and rehabilitation lead to a more normal bacterial community dominated by Bacteroidetes and Firmicutes. If this interpretation is correct, then the assumption that lengthy stays in captivity or rehabilitation centers cause dysbiosis does not hold firm for all cases, especially for turtles suffering from illness that may be secondarily altering the gut microbiota. Biagi et al. also found that time in a rehabilitation center did not impact any feature of the gut microbiota (alpha diversity, beta diversity or RA) in loggerheads (Biagi et al., 2019).

While it was found that in green turtles (Campos et al., 2018) and Kemp's ridleys (Samuelson et al., 2020) Bacteroidetes are more abundant than Firmicutes, Arizza et al. found the opposite was true in loggerhead sea turtles (Arizza et al., 2019). Across 9 fecal samples sequenced from live stranded loggerhead sea turtles, Firmicutes was identified as the dominant phylum with a RA of 49.4% (Arizza et al., 2019). Bacteroidetes (RA 21.5%) was the second most abundant phylum and Proteobacteria (RA 11.0%) the third (Arizza et al., 2019). Two additional studies also found Firmicutes as the most dominant phylum in loggerhead sea turtles (Abdelrhman et al., 2016; Biagi at al., 2019). The first,

obtained 58 fecal samples from 29 live loggerheads stranded or captured by fishery net that were housed for rehabilitation (Biagi at al., 2019). Among these samples, the major phyla identified included Firmicutes (RA 46.5%), Fusobacteria (RA 26.5%), Bacteroidetes (RA not reported), and Proteobacteria (RA not reported) (Biagi et al., 2019). The second study sampled 4 hospitalized loggerheads following stranding (Abdelrhman et al., 2016). Firmicutes was the most abundant phylum with a RA of 66.0%, followed by Proteobacteria (RA 23.0%) and Bacteroidetes (RA 6.2%) (Abdelrhman et al., 2016). The shift in dominance from Bacteroidetes to Firmicutes in loggerhead sea turtles in comparison to green sea turtles has been suggested to be secondary to differences in diet between the species (Arizza et al., 2019). Adult green turtles eat a primarily herbivorous diet, while loggerheads from post-hatching into adulthood are carnivorous feeders (Hoopes et al., 2017; Arizza et al., 2019). Though, if diet were the driving force, you would expect the gut microbiota of the Kemp's ridley sea turtle would more closely resemble that of the loggerhead sea turtle as their diets are more closely aligned (Hoopes et al., 2017).

Patterns of microbiota in fecal samples across studies and between species are more difficult to identify at lower levels of taxonomic classes. These difficulties arise from differences in methodology, collection time, and populations sampled. However, regardless of these challenges, a few taxonomic classes and families have been identified as abundant in multiple studies. The family identified across the most studies was Clostridiaceae (Abdelrhman et al., 2016; Campos et a., 2018; Biagi et al., 2019; Arizza et al., 2019; Ahasan et al., 2019; Bloodgood et al., 2020). Clostridiaceae has been identified as an abundant family in wild, and captive green sea turtles (Kittle et al., 2018; Campos et al., 2018; Ahasan et al., 2019). Bloodgood et al. reported that Clostridiaceae and an unclassified family from the order Clostridiales were both significantly more abundant at the time of admission to rehabilitation than at mid-rehabilitation or recovery time points (Bloodgood et al., 2020). Clostridiaceae has also been identified as a prominent family in loggerheads (Abdelrhman et al., 2016; Biagi et al., 2019; Arizza et al., 2019). Abdelrhman et al. reported that the Firmicutes phylum was dominated by the class Clostridia with the most represented genera, Clostridium XI (RA 21.3%) and *Clostridium sensustrict* (RA 14.6%), falling within the family Clostridiaceae (Abdelrhman et al., 2016). Additionally, Biagi et al. reported a 17.8% RA of Clostridiaceae with the *Clostridium* genus having a RA of 14.8% (Biagi et al., 2019). Other commonly identified families of bacteria reported at differing RA's across fecal studies include Bacteroidaceae, Lachnospiraceae, Ruminococcaceae and Fusobacteriaceae (Abdelrhman et al., 2016; Kittle et al., 2018; Campos et al., 2018; Biagi et al., 2019; Arizza et al., 2019; Samuelson et al., 2020). While the studies mentioned may not agree exactly on the relative abundance of each phylum, class, family, or genus of bacteria in the sea turtle's feces, there is an emerging pattern suggesting that a normal core microbiota of sea turtles likely exists. Continued investigation of the fecal microbiota in healthy turtles, with a more uniform approach, would help illuminate these developing patterns.

There are multiple factors that may alter the microbiota detected in the feces of sea turtles, including but not limited to health status, diet, age, location, treatment, and debris (Ahasan et al., 2019; Scheelings et al., 2020b; Biagi et al., 2021). Ahasan et al.

investigated the effect of antibiotics versus bacteriophage therapy on the fecal microbiota of juvenile green turtles raised in captivity from emergence (Ahasan et al., 2019). The dominant phyla identified across all treatment and control groups were Firmicutes, Bacteroidetes and Proteobacteria. Within these phyla, the genera *Clostridium* and *Bacteroides* were identified as the most predominant. Bacteriophage treated turtles were observed to have transient changes to their fecal microbiota with a specific decrease in the targeted microbe, while treatment with enrofloxacin resulted in a gradual decrease in fecal diversity, which did not recover immediately after discontinuing the antibiotic. Antibiotic treatment resulted in an increase in the phylum Firmicutes, and more specifically, an increase in microbes within the class Clostridia. Also observed were small decreases in the abundance of previously dominant phyla including Bacteroidetes, Proteobacteria, Verrucomicrobia, and Actinobacteria. It is important to note that this study took hatchlings immediately following emergence from the nest and raised them in a lab for 11 months (Ahasan et al., 2019). Lack of exposure to the normal oceanic environment and diet during the early stages of life may have innately changed what is considered normal microbiota in the control group. Regardless of if the normal microbiota identified are equivalent to the wild counterparts, treatment with a broad-spectrum antibiotic, such as enrofloxacin, has the potential to alter the ratios of dominant bacterial phyla within the fecal microbiota. Additionally, the resulting alterations in fecal microbiota do not quickly return to normal levels following discontinuation of the antibiotic (Ahasan et al., 2019).

Ingestion of foreign items is a threat to sea turtles and is likely the result of indiscriminate feeding or mistaking foreign objects as food, as such there is growing concern about the effect that plastic may have after ingestion (Boylan et al., 2017a). Ingested plastic debris can act as a vehicle for microbes, chemical pollutants, and toxic compounds (Boylan et al., 2017a; Biagi et al., 2021). Additionally, plastic debris have the potential to cause epithelial damage to the gastrointestinal tract during passage and can result in local inflammation, which may alter the environment within the gastrointestinal tract (Biagi et al., 2021). Biagi et al. 2021 investigated the effect of plastic debris on the fecal microbiota of 45 stranded or captured loggerhead sea turtles in the Northwestern Adriatic Sea (Biagi et al., 2021). Forty-eight operational taxonomic units (OTUs) were noted to be increased in the presence of plastic debris in the feces. The most dominant species identified within this group was *Cetobacterium somerae* belonging to the phylum Fusobacteria. While this species of bacterium was previously noted to be normal fecal microbiota of loggerheads by Biagi et al. in 2019, the relative abundance of the microbe was notably increased in the presence of plastic debris (Biagi et al., 2019; Biagi et al., 2021). Other genera that increased with an increase in plastic debris included *Fusobacterium, Vibrio, Psychrobacter, Romboutsia, Desertihabitans, Staphylococcus, Terriporobacter,* and *Gordonibacter* (Biagi et al., 2021). A number of genera were seen to decrease in abundance with an increase in plastic debris. Genera in this category included *Clostridium, Faecalicatena, Akkermansia, Rikenella, Cloacibacillus, Pseudoflavonifractor,* and *Romboutsia* (Biagi et al., 2021). Plastic debris appears to be correlated with changes to the fecal microbiota and should be kept in mind when sampling feces in wild sea turtles for gastrointestinal microbiota studies, especially in

areas where plastic pollution is high (Biagi et al., 2021). Accordingly, health status, diet, age, location, medical treatment, and plastic debris should be accounted for when investigating and interpreting the fecal microbiota of sea turtles.

Distal colon swabs

Distal colon swabs (i.e., swabs that enter via the cloaca and are extended a minimum of 60 cm into the distal colon) are the best method currently available for sampling the gastrointestinal microbiota in healthy living sea turtles (Scheelings et al., 2020a). While this method does not allow for understanding of individual sections of the gastrointestinal tract, it is likely less affected by outside factors such as water and sand (Scheelings et al., 2020a). Thus, it is an ideal way to identify the normal microbiota in healthy sea turtles and can aid in the interpretation of post-mortem samples collected. Comparisons between live distal colon swab samples and post-mortem gastrointestinal samples will allow researchers to identify changes in the microbiota that occurred post-mortem and those that occurred anti-mortem and may be due to significant pathology.

Using this advantageous technique on healthy adults, Scheelings et al. investigated the gut microbiota of geographically distinct populations of nesting female loggerhead and flatback sea turtles (*Natator depressus*) (Scheelings et al., 2020a). The loggerheads sampled included 6 from Florida, USA and 18 from Queensland, Australia. The flatbacks included 19 from Crab Island, Queensland Australia and 10 from Port Hedland, Western Australia. Across all loggerheads sampled the most abundant phylum was Proteobacteria. The other prominent phyla, identified from the US population in order of abundance included Actinobacteria, Bacteroides, and Firmicutes. In contrast, the dominant phyla identified from loggerheads in Australia included Spirochaetes, Bacteroides, and Actinobacteria. Between the two locations, 37 OTUs were shared, but each location had unique OTUs not identified in the other location. Twenty-seven OTUs were unique to the US turtles and 61 were unique to the turtles sampled in Australia. The alpha diversity was noted to be significantly different between the two populations of loggerheads based on location. A similar trend of differences based on location was seen in the two flatback populations. The predominant phyla in turtles sampled from Crab Island was Firmicutes, while Proteobacteria was the most commonly identified phylum from the turtles sampled at Port Hedland. There were 28 OTUs shared between the two flatback populations, but again each location had unique OTUs. Five OTUs were identified as unique to Crab Island and 61 were unique to Port Hedland. Alpha diversity was again noted to be significantly different between the two populations. This study suggested that geographic location plays a role in shaping the core microbiota of the gastrointestinal tract. It is important to note that additional factors, such as foraging location, could not be accounted for in this study. For example, nesting females often return to the same nesting beach, but each beach serves as a nesting ground for females from numerous foraging locations (Scheelings et al., 2020a).

In 2020, Scheelings et al., also reported microbiota data obtained from nesting females of all seven extant sea turtle species via distal colon swabs (Scheelings et al., 2020b). The most abundant bacterial phyla identified across all species included Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes. Kemp’s ridleys had three

phyla identified that were found in no other species: Euryarchaeota, Deferribacteres, and Cyanobacteria. There were clustering patterns of beta diversity among the leatherbacks and green turtles, which are the more ancient species. Regardless of species, Proteobacteria was the most predominant phylum identified in the colon of all sea turtles. Given this common finding among all species, Scheelings et al. suggested that if Proteobacteria is not the most abundant phylum in a nesting female it should be considered abnormal. This statement seems to be in direct contrast to those made by Campos et al. and Samuelson et al. stating that an increase in Proteobacteria is associated with dysbiosis (Campos et al., 2018; Samuelson et al., 2020). It is important to note that nesting female turtles undergo prolonged periods of anorexia (Scheelings et al., 2020b). This anorexia may lead to a normal shift in the gut microbiota that results in an increased abundance of Proteobacteria. Campos et al. and Samuelson et al. sampled turtles at a different life-stage classes and physiologic stage than that of a nesting female turtle (Campos et al., 2018; Samuelson et al., 2020). At certain life-stage classes, a shift in microbiota to Proteobacteria may be indicative of dysbiosis, while in a nesting female it may be a normal change secondary to prolonged inappetence during the nesting season.

Further investigation in healthy sea turtles at all life-stage classes will be important for identifying the core microbiota of each species. Broad application of distal colon swabs assessed via next generation sequencing in healthy sea turtles will tremendously expand our knowledge of the sea turtle gastrointestinal microbiota. Expansion of baseline microbiota data in healthy individuals will allow definitive identification of a core gastrointestinal microbiota. Once this core is identified that knowledge can then be used and applied to husbandry, treatment, and disease prevention in sea turtles both in the wild and in captivity.

Mucosal Surfaces

Mucosal surfaces such as the cloaca, oral cavity, and nasal cavity are prime environments to support microbial communities (Neish et al., 2014). The normal flora at these locations often serves as a primary defense against opportunistic and pathogenic bacterial colonization (Neish et al., 2014). Mucosal surfaces are located at the interface of the host and the environment and therefore can generally be easily accessed and sampled from living animals (Neish et al., 2014). Uniquely, mucosal surfaces are generally in constant contact with the aquatic environment that sea turtles live in (Price et al., 2017). Consequently, changes in mucosal microbiota may be affected by changes in the environment such as increased temperature, pH, and pollution (Al-Bahry et al., 2011; Apprill et al., 2017). These alterations in microbiota could be detected and used as evidence that a pollutant is present and negatively impacting normal physiology of sea turtles (Bahry et al., 2011). Additionally, many sea turtle mucosal surfaces are contiguous with the gastrointestinal tract and can be used to help understand the gastrointestinal microbiota (Price et al., 2017; Ahasan et al., 2020). More specifically, cloacal samples are often used to make inferences about gastrointestinal microbiota but are more accurately grouped as a part of external mucosal surfaces (Abdelrhman et al., 2016; Price et al., 2017; Ahasan et al., 2018; Ahasan et al., 2020). As such, this section includes the

current studies investigating the microbiota of the nasal cavity, cloaca, and the oral cavity.

Nasal samples

Several studies have utilized traditional culture methods combined with biochemical tests to identify the bacteria of the nasal cavity, but they are difficult to interpret as they compare the results to cloacal samples and do not report the results independently from each sample location (Santoro et al., 2006; Santoro et al., 2008; Zavala-Norzagaray et al., 2015). In 2006, Santoro et al. used culture to isolate and identify the nasal and cloacal microbiota of nesting green turtles (Santoro et al., 2006). The gram-positive isolates were predominately composed of *Staphylococcus* species, which made up 73.2% of all gram-positive microorganisms. Of the gram-negative isolates, 53.1% of them belonged to the Enterobacteriaceae family, including the genera *Enterobacter* sp., *Escherichia* sp., *Klebsiella* sp. and *Serratia* sp. The most identified microorganism from all samples was *Klebsiella pneumoniae*, with a 47.1% and 38.5% prevalence in nasal and cloacal samples respectively (Santoro et al., 2006). In 2008, Santoro et al. performed another culture-based study to identify the nasal and cloaca microbiota of nesting female leatherback sea turtles (*Dermochelys coriacea*) (Santoro et al., 2008). Similarly, this study reported that a predominant portion of the gram-negative isolates (60/113) fell within the Enterobacteriaceae family. Isolates included species from the genera *Enterobacter* sp., *Escherichia* sp., *Klebsiella* sp., *Proteus* sp., and *Salmonella* sp. (Santoro et al., 2008). Contrary to the gram-positive profile proposed in the green sea turtles (Santoro et al., 2006), 6 out of the 10 gram-positive isolates from the cloaca of leatherbacks were identified as *Enterococcus faecalis* (Santoro et al., 2008). Also isolated from the nasal cavity and cloaca of leatherbacks were *Bacillus* sp., *Aeromonas* sp., *Pseudomonas* sp., and *Vibrio* sp. (Santoro et al., 2008). Both *Aeromonas* sp. and *Pseudomonas* sp. were identified more frequently in the cloaca, while *Bacillu*s sp. was more prominent in the nasal cavity. Of the *Bacillus* sp. isolates, 96.3% came from the nasal samples (Santoro et al., 2008). In a culture-based investigation for antibiotic resistant *Vibrio* spp., Zavala-Norzagaray et al. identified microbiota from the nasopharynx and cloaca of green and olive ridley (*Lepidochelys olivacea*) sea turtles (Zavala-Norzagaray et al., 2015). The identified microorganisms were again dominated by gram-negative bacteria within the Enterobacteriaceae family, including species within the genera *Citerbacter* sp., *Escherichia* sp., *Edwarsiella* sp., *Morganella*/*Proteus* sp., and *Providencia* sp. In addition to these genera, numerous species of *Vibrio* sp. were identified. Isolates found solely in the cloaca included *Citerobacter freundii*, *E. coli*, *Morganella*/*Proteus*, *Providencia*, and *Vibrio fluvialis*. *Vibrio alginolyticus*, *V. cholera*, and *V. parahaemolyticus* were isolated from both the nasopharynx and the cloaca. Isolates found only in the nasopharynx included *Edwarsiella* spp., *Vibrio furnisii*, and *Vibrio* spp. (Zavala-Norzagaray et al., 2015).

Although differences in methods make it difficult to draw conclusions across studies, it is apparent that each mucosal surface has a unique microbiota with some potential overlap. These comparison studies make it increasingly evident that clearly separating each anatomic location during analysis is necessary to get the most accurate

understanding of the core microbiota at each site. Additionally, unifying methodology across studies aiming to identify core microbiota will allow a broader picture to be drawn across all anatomic locations and species of sea turtles.

Cloacal samples

Keene et al. investigated cloacal samples from green and olive ridley sea turtles and cultured microbiota similar to those previously mentioned (Keene et al., 2014). Cloacal samples were dominated by gram-negative bacteria in the Enterobacteriaceae family. Within this family *Serratia* sp., *Enterobacter* sp., *Klebsiella* sp., and *Salmonella* sp. were isolated. Gram-positive isolates commonly identified included species within the genera *Corynebacteria* sp., *Bacillus* sp., and *Staphylococcus* sp. Nine bacteria were isolated from cloacal fluid samples during this study and were not found in sand samples. The bacteria unique to the cloaca included *Pseudomonas aeruginosa*, *Serratia plymuthica, Enterococcus faecalis, Enterobacter cloacae, Klebsiella* sp., *Salmonella* sp., *Bacillus* sp., *Staphylococcus epidermidis*, and *Enterobacter sakazakii* (Keene et al., 2014).

Culture based investigations of the cloaca of loggerhead sea turtles have also been performed and reveal similar microbial profiles to those found in green turtles and olive ridleys (Alduina et al., 2020; Blasi et al., 2020). Blasi et al. screened for gram-negative bacteria via cloacal swabs in 33 wild caught juvenile loggerheads (Blasi et al., 2020). Of the 33 turtles sampled, 23 were classified as healthy and 10 individuals were classified as weak based on physical examination. Ninety enteric microbes were cultured and isolated from all 33 swabs; of them 59% of the isolates belonged to the family Enterobacteriaceae and 31% belonged to the family Shewanellaceae. Due to the nature of this study, comparisons between the healthy and weak groups of turtles were made and unique genera were identified within each group. *Hafnia, Achromobacter, Leclercia,* and *Proteus cibarius* were isolated only from turtles categorized as healthy. *Acinetobacter*, *Kluyvera intermedia,* and *Serratia* were identified only in turtles grouped as weak. It was also stated that *Klebsiella, Morganell*a, and *Providencia* were notably more prevalent in healthy turtles. Additionally, a list of core bacteria was identified across all turtles, though at varying amounts within individuals. The core microbiota included *Shewanella* sp., *Klebsiella oxytoca*, *Morganella morganii*, *Providencia rettgeri, Enterobacter* spp., *Citrobacter freundii, Pseudomonas* sp., and *Vibrio* spp. (Blasi et al., 2020).

In 2020, Alduina et al. performed a broad study to identify antibiotic resistant bacteria in loggerheads in the Mediterranean Sea (Alduina et al., 2020). The study included swabbing the organs of 8 fresh dead turtles and swabs from the cloaca, oral cavity, and skin from 14 loggerheads upon arrival for rehabilitation. The samples were cultured, and isolates were identified using a combination of biochemical enzymatic tests and sequencing. Across all samples, the most abundant organisms isolated were *Aeromonas* spp., *Citrobacter* spp., and *Enterobacter* spp. Among the 8 cloacal samples taken, several genera within the family Enterobacteriaceae, including *Citerobacter* sp., *Enterobacter* sp., *Escherichia coli,* and *Klebsiella* sp., were isolated. *Pseudomonas* spp. and *Aeromonas* were also isolated from cloacal samples (Alduina et al., 2020). Across the studies utilizing culture methods to identify the microbiota within the cloaca, bacteria

within the family Enterobacteriaceae appear to dominate across all three species of sea turtles investigated thus far (Keene et al., 2014; Alduina et al., 2020; Blasi et al., 2020). Though, it should be noted that the two studies performed on loggerheads (Alduina et al., 2020; Blasi et al., 2020) were focused more on identifying antibiotic resistant bacteria. This focus likely created a bias in the culture results towards gram-negative bacteria.

In addition to the previously mentioned studies utilizing traditional culture techniques, there are also an array of studies that have used high throughput sequencing to identify the microbiota on the cloacal surface of sea turtles. Three of these studies collected cloacal samples from wild captured green turtles (Ahasan et al., 2017; Price et al., 2017; McNally et al., 2021b), two of which found similar profiles with Firmicutes and Bacteroidetes as the dominant phyla (Ahasan et al., 2017; Price et al., 2017). Price et al. investigated the cloacal microbiota in wild caught juvenile green turtles from three different habitats (pelagic, beachfront, bay) in the Northern Gulf of Mexico (Price et al., 2017). Eighteen cloacal swabs were obtained and Bacteroidetes and Firmicutes composed 30% of all bacteria identified from all three habitats. Firmicutes was represented almost entirely by the class Clostridia, with the family Ruminococcaceae being found solely in turtles from pelagic habitats and the family Lachnospiraceae being found only in turtles from bay habitats (Price et al., 2017).

Similar dominant phyla were identified in 8 deep cloacal swabs from wild-caught green turtles on the Great Barrier Reef (Ahasan et al., 2017). Ahasan et al. reported Firmicutes as the most abundant phyla with a RA ranging from 60.5–62.6% and Bacteroidetes with a RA ranging from 27.6–31.9% (Ahasan et al., 2017). In this same study, Ahasan et al. also performed cloacal swabs on 4 stranded green sea turtles from the Great Barrier Reef. Samples from stranded green sea turtles were dominated by Proteobacteria (RA 47.6%), followed by Bacteroidetes (RA 19.0%), Firmicutes (RA 18.7%), and Fusobacteria (13.6%). Statistical comparisons were made between the wild caught and stranded turtles and a significant difference in bacterial diversity was identified. A higher RA of Proteobacteria was associated with stranded turtles (Ahasan et al., 2017). A high abundance of Proteobacteria was also reported by Ahasan et al. in 2018 during an investigation of 4 hospitalized green turtles before and after rehabilitation (Samuelson et al., 2020). At both pre-hospitalization and post-rehabilitation, Proteobacteria was the most dominant phylum. Prevalent phyla were determined via calculating cumulative abundance (CA). Phyla in order of most abundant to least from pre-hospitalization samples were Proteobacteria (CA 33.6%), Firmicutes (CA 25.5%), Bacteroides (CA 14.4%), and Fusobacteria (CA 9.1%). Cumulative abundance of each phylum post-rehabilitation was Proteobacteria 36.9%, Bacteroidetes 25.4%, Fusobacteria 16.1%, and Firmicutes 14.2% (Ahasan et al., 2018).

In direct contrast to the two other studies on wild-caught green turtles, Ahasan et al., 2017; Price et al., 2017), McNally et al. 2021 found an abundance of microbial families within the Proteobacteria phylum in wild-caught green turtles (McNally et al., 2021b). Twenty green turtles were caught off the coast of Florida adjacent to the St. Martins Marsh Aquatic Preserve of Crystal River. Among all 20 samples the families Neisseriaceae and Arcobacteraceae were found to have the highest percent mean abundance (MA) at 29.2% and 14.7% respectively (McNally et al., 2021b). These

findings are more comparable to samples obtained from stranded and hospitalized green turtles (Ahasan et al., 2017; Ahasan et al., 2018) and brings forth the question, what external factors may be affecting cloacal microbiota?

In 2021, McNally et al. also used high throughput sequencing to investigate the cloacal microbiota of 30 wild caught Kemp's ridleys in St. Martins Marsh Aquatic Preserve (McNally et al., 2021b). The most abundant families identified, and their corresponding percent mean abundances (MA) were Cardiobacteriaceae 16.5%, Flavobacteriaceae 15.5%, and Neisseriaceae 10.4%, all of which fall within the phyla Proteobacteria or Bacteroidetes. Also in 2021, McNally et al. investigated the cloacal microbiota of cold stunned Kemp's ridleys (McNally et al., 2021a). Cloacal samples were collected at intake to the New England Aquarium and again when the animal was declared clinically healthy. The determination of clinically healthy for turtles not treated with antibiotics was defined as ready for release and was based on appetite, physical examination, and transport ability. For turtles treated with antibiotics, clinical health was defined as 30 days past the termination of antibiotic treatment. The most prominent family identified at intake was Vibrionaceae with a mean abundance of 23.1%. The other three predominant families identified at intake were Arcobacteraceae, Shewanellaceae, and Rhodobacteraceae with mean abundances of 11.8%, 7.7%, and 6.7% respectively. All four of the predominant families fell within the phylum Proteobacteria (McNally et al., 2021a). In contrast, in clinically healthy Kemp's ridles, there was an increase in the family Flavobacteriacea (MA 17%), making it the most abundant bacterial family. The other predominant families identified in clinically healthy individuals were Vibrionaceae (MA 13.6%), Arcobacteraceae (MA 10.3%), and Rhodobacteraceae (MA 9.1%). Like in wild caught Kemp's ridleys, the most predominant bacterial families of clinically healthy individuals all fell within the phyla Bacteroidetes or Proteobacteria (McNally et al., 2021a). Additionally, McNally et al. identified a trend of increasing Shannon diversity from intake to the time that an animal was identified as clinically healthy (McNally et al., 2021a). Consequently, this study concluded that the microbiota of cold-stunned Kemp's ridley sea turtles can be affected by several factors including but not limited to disease status, local environment, and antibiotics (McNally et al., 2021a).

Across studies using next generation sequencing on cloacal samples, there appear to be emerging patterns of microbiota within sea turtle species, location, and health status. Further investigations into sub populations of sea turtles will continue to delineate these patterns. It should be highlighted as a note of caution that cloacal samples are often used to make inferences about the gastrointestinal microbiota of sea turtles. While this can give an idea of common enteric microorganisms found in this area, it is influenced by multiple environmental factors due to a turtle's constant emersion in water (Price et al., 2017). Price et al. suggested that the microbiota of the cloaca appeared to be more influenced by environment than by the gut (Price et al., 2017). Additionally, it is becoming clear that microbiota of any given site is influenced by many other factors such as age, location, and disease status (McNally et al., 2021a). For this reason, broad inferences between populations or to gut microbiota must be made with caution and should take into consideration the other factors that may have a role in the establishment of the cloacal microbiota.

Oral samples

Oral microbiota in sea turtles have been investigated through both culture and next generation sequencing methods. In 2020, Alduina et al. obtained 6 oral swabs from loggerheads upon arrival for rehabilitation (Alduina et al., 2020). Through culture techniques *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., *Citerobacter*, and *Aeromonas* were all identified in the buccal cavity (Alduina et al., 2020).

In 2021, McNally et al. investigated oral microbial communities in wild-caught green turtles and Kemp's ridleys (McNally et al., 2021b). Within the oral cavity of green turtles, four predominant families of bacteria were identified, with three falling under the phylum Proteobacteria, including Pasteurellaceae (MA 44.8%), Arcobacteraceae (MA 15.6%), and Campylobacteraceae (MA 9.9%). In contrast, the most predominant family in Kemp's ridleys was Flavobacteriaceae (MA 34.8%), which falls under the phylum Bacteroidetes. After Flavobacteriacea, Arcobacteraceae (MA 11.6%) was the next most abundant family followed closely by Rhodobacteraceae (MA 8.7%) (McNally et al., 2021b).

Similarly, McNally et al. also found that Flavobacteriaceae had the highest mean abundance of all bacterial families when sampling the oral microbiota of cold stunned Kemp's ridleys at intake and then again when clinically healthy (McNally et al., 2021a). Mean abundance of Flavobacteriaceae was reported as 30% at intake and 22.5% after being declared clinically healthy. There were differences in predominant families and their reported mean abundance for oral samples taken at intake versus those taken from individuals deemed clinically healthy. At intake, the other dominant families were reported to be Rhodobacteraceae (MA 13.7%), Vibrionaceae (MA 9%), and Porticocaceae (6%) (McNally et al. 2021a). In contrast, in turtles that were deemed to be clinically healthy, Rhodobacteriae had an increased mean abundance at 20.6%, followed by the presence of an unassigned Gammaproteobacteria family (MA 12.1%), and Saprospiraceae (MA 10.8%) (McNally et al. 2021a). Regardless of health status or captivity of Kemp's ridleys Flavobacteriaceae appears to make up an essential portion of the oral microbiota. Furthermore, the oral microbiota is persistently dominated by bacterial families falling within the phyla Bacteroidetes and Proteobacteria in all species sampled thus far (McNally et al. 2021a; McNally et al. 2021b).

Skin and shell

Investigations into the microbiota on the skin of sea turtles are lacking. Currently there is only one study reporting skin data from 2 turtles (Alduina et al., 2020). The skin is an incredibly important organ as it is an animal's primary barrier and defense against the outside world (Boylan et al., 2017b). In sea turtles, the skin is also in constant direct contact with their aquatic environment making it of great interest. In hard-shelled (cheloniid) sea turtles the epidermal-dermal unit is categorized into two groups, scutes and scales (Boylan et al., 2017b). The scutes are thick stratum corneum that form plates to make up the shell (Boylan et al., 2017b). The shell has 3 distinct regions, the carapace, the plastron, and the bridge (Boylan et al., 2017b). The scales cover the rest of the body including the head, neck, and appendages (Boylan et al., 2017b). The differing microscopic composition of the skin in these locations is important to consider when

investigating the microbiota of the skin. Different regions of the shell, head, and appendages may be home to different microbiota. Additionally, the leatherback sea turtle (*Dermochelys coriacea*) has a skin composition unique from all other sea turtle species and lacks prominent scalation and heavy cornification (Boylan et al., 2017b). Consequently, leatherback skin microbiota should be investigated on its own as it may be highly different from other sea turtle species.

The previously mentioned study performed by Alduina et al. in 2020 included skin swabs from 2 loggerhead sea turtles upon arrival for rehabilitation (Alduina et al., 2020). Traditional culture techniques were used and isolated *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., and *Aeromonas* (Alduina et al., 2020). There is no current literature citing the use of next generation sequencing on skin samples in sea turtles. However, there are studies that investigated the skin and shell microbiota of freshwater turtles such as the red-eared slider (*Trachemys scripta*) and the Krefft's river turtle (*Emydura macquarii krefftii*) (Parks et al., 2020; McKnight et al., 2020). These studies suggest that there are unique microbiota located on different parts of the skin, such as the head and the shell with and without algae (Parks et al., 2020; McKnight et al., 2020). Microbiota located on the skin, plastron, and carapace of sea turtles is an area in need of investigation. Additionally, unique consideration should be given to regions of the skin that are colonized with epibiota and algae. The concentrations of other organisms, both commensal and parasitic, could alter the core microbiota of the skin at a given location (Parks et al., 2020; McKnight et al., 2020).

Sand, Nest, and Eggs

Summarizing all microbiota data from sand, nests, and eggs to date is beyond the scope of this review as this review aims to summarize the microbiota found on and within the sea turtle. However, it is important to note that studies investigating the presence of microbes in the nest and on the outside of sea turtle eggs do exist but are often performed on dead eggs (Wyneken et al., 1988). As such, there is a high likelihood of contamination from sand, humans, predators, and other unknown variables that make the data difficult to interpret. Additionally, unhatched eggs found after emergence are not a good representation of what would be found on the outside of a normal and healthy egg prior to hatching. An investigation of the outer microbiota of the eggs as soon as they are released from the mother would be beneficial. Currently, there are some studies that sample eggs as they fall from the cloaca, but they often focus on pathogen identification as opposed to identification of core microbiota (Al-bahry et al., 2009; Sarmiento-Ramírez et al., 2014, Rosado-Rodríguez et al., 2016). There is still a large knowledge gap regarding the core microbiota of the sea turtle egg. A study using next generation sequencing, aimed at identifying the normal microbiota on the egg's surface could provide insight as to where the core microbiota, both skin and gastrointestinal, come from. Furthermore, identifying the source of the microbiota may help labs and rehabilitation centers support a more natural development of core microbiota in hatchlings and post hatchlings.

Conclusion

In the last few years high throughput sequencing has significantly aided the advancement in baseline knowledge of microbiota in sea turtles. Specifically, the knowledge of core microbiota within the gastrointestinal tract of sea turtles has received a significant amount of attention and advancement because of the increasing access and affordability of sequencing technology. The next step for the gastrointestinal microbiota research is to shift aims at testing hypotheses to produce meaningful and applicable clinical knowledge for sea turtles. Such knowledge could include methods of detecting and treating dysbiosis in sea turtles, development of pre- and probiotics for captive animals, determination of the least harmful but still effective antimicrobial therapy and identifying relationships between the gastrointestinal microbiota and important physiologic processes. However, while there have been great advances in baseline gastrointestinal tract microbiota data, there are still other large knowledge gaps within baseline microbiota research in sea turtles.

Knowledge of the skin microbiota is one such gap, with little to no research. Filling this gap and identifying core skin microbiota data may pave the way for development of topical skin pre- and probiotics for sea turtles. Furthermore, this knowledge could aid in the treatment of common skin issues in sea turtles such as infections, open wounds, or fibropapillomatosis. As for free ranging turtles, the skin's constant contact with their environment and thus the contaminants within it may provide insight into what the turtles are being exposed to. Alterations in core microbiota may be associated with exposure to specific chemical pollutants or toxins in the water. Identification of such patterns will allow researchers and conservationists to detect contaminant exposure and may aid in the development of species-specific mitigation plans, conservation efforts, and policy changes to prevent future exposure. Additionally, specific alterations in skin microbiota could be detected in conjunction with changes in ocean temperatures and may provide additional insight into the effects of climate change on these threatened animals. Another knowledge gap exists regarding the microbiota found on external surfaces of healthy sea turtle eggs. Knowledge of what is found on healthy eggs could be used evaluate the effect of rising beach temperatures on otherwise healthy eggs and could potentially be a tool used to predict reproductive success. Moreover, investigating ways to influence success of a nest by manipulating the microbiota on the egg is an additional possibility.

Although methodological differences make it challenging to draw conclusions across studies, it is apparent that each anatomical location has a unique core microbiota with some potential overlap. Unifying methodology across microbiota studies will allow a broader picture to be drawn across all anatomic locations and species of sea turtles. Furthermore, such unity will aid with the application of the research, both clinically and in the development of conservation efforts. Finally, this review focused on bacterial communities found on and within sea turtles, but those are not the only types of microorganisms that can colonizes these regions. Future studies should investigate these same locations for the presence of a wider range of organisms including fungi, viruses, parasites, epibiota, and archaea. Including additional types of organisms in microbiome

work can unveil relationships among the organisms and between the organisms and the host that are essential for the maintenance of a healthy microbiome.

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Appendix

Table 1.1 Publications on sea turtle microbiota**.** Included are the references, species of turtle(s) studied, the state of the animal(s), method of identification, time of sample, and sample type.

Reference	**Species**	**Status**	**Identification Method**	**Sample timeline**	**Sample(s)**
Kittle et al. 2018	*Chelonia mydas*	Euthanized	Culture	Post-mortem	Gastrointestinal tract
McDermid et al. 2020	*Chelonia mydas*	Euthanized	Culture	Post-mortem	Gastrointestinal tract
Ahasan et al. 2020	*Chelonia mydas*	Stranded and died 1-4 days after arrival to rehab center	Next generation sequencing	Post-mortem	Oesophagus, stomach, small intestines, and large intestines
Campos et al. 2018	*Chelonia mydas*	Captive, wild caught and recently dead	Next generation sequencing	Wild caught: confined to tanks for 24-36 hours until the defecated	Fecal
Bloodgood et al. 2020	*Chelonia mydas*	Live in rehabilitation center	Next generation sequencing	Sampled at 3 time points: admission, mid-rehabilitation, and recovery	Fecal
Abdelrhman et al. 2016	*Caretta caretta*	Life in rehabilitation center or recently dead	Next generation sequencing	Live samples occurred between 21 to 41 days of rehabilitation	Fecal and cloaca
Arizza et al. 2019	*Caretta caretta*	Stranded and admitted for rehabilitation	Next generation sequencing	First fecal sample after animal recovery (2-78 days of rehabilitation)	Fecal
Biagi et al. 2019	*Caretta caretta*	Live Stranded/captured by fishery nets and admitted for rehabilitation	Next generation sequencing	One sample close to feeding time and the other distant (2-3) days after feeding but before next meal. Samples were taken between 6 and 210 days of rehabilitation	Fecal
Biagi et al. 2021	*Caretta caretta*	Live Stranded/captured by fishery nets and admitted for rehabilitation	Next generation sequencing	First fecal sample after arrival to rehabilitation center	Fecal
Samuelson et al. 2020	*Lepidochelys kempii*	Incidentally captured and housed for rehabilitation	Next generation sequencing	Samples were collected right before release and sorted into short term and long-term stays.	Fecal

Table 1.1 continued

Reference	Species	Status	Identification Method	Sample timeline	Sample(s)
Ahasan et al. 2019	*Chelonia mydas*	Captive raised	Next generation sequencing	Euthanized at day 6, 15 and 28 after being in a treatment group (control, antibiotic, bacterial phage)	Fecal
Scheelings et al. 2020b	All 7 extant species	Nesting females	Next generation sequencing	Sampled at nesting event	Distal colon swabs
Scheelings et al. 2020a	*Caretta caretta* and *Natator depressus*	Nesting females	Next generation sequencing	Sampled at nesting event	Distal colon swabs
Santoro et al. 2008	*Dermochelys coriacea*	Nesting females	Culture	Sampled at nesting event	Cloaca and nasal
Zavala-Norzagaray et al. 2015	*Chelonia mydas agassizii* and *Lepidochelys olivacea*	Live in water capture	Culture	Sampled at capture	Cloaca and nasopharynx
Santoro et al. 2006	*Chelonia mydas*	Nesting females	Culture	Sampled at nesting event	Cloaca and nasal
Keene et al. 2014	*Chelonia mydas agassizii* and *Lepidochelys olivacea*	Nesting females	Culture	Sampled at nesting event	Cloaca, eggs, and sand
Alduina et al. 2020	*Caretta caretta*	Live in rehabilitation center	Culture for isolation and Next generation sequencing for identification	Sampled upon arrival for rehabilitation	Cloaca, oral, and skin
McNally et al. 2021a	*Lepidochelys kempii*	Live in rehabilitation center	Next generation sequencing	Sampled at intake and again when deemed clinically healthy	Cloaca and oral
McNally et al. 2021b	*Lepidochelys kempii* and *Chelonia mydas*	Live in water captures	Next generation sequencing	Sampled at capture	Cloaca and oral
Blasi et al. 2020	*Caretta caretta*	Live in water captures: classified as healthy or weak	Culture for isolation and sequencing for identification	Sampled at capture	Cloaca
Ahasan et al. 2017	*Chelonia mydas*	Wild captured and stranded	Next generation sequencing	Wild captured were sampled at capture. Stranded turtles were sampled upon arrival for rehabilitation	Cloaca
Ahasan et al. 2018	*Chelonia mydas*	Live in rehabilitation center	Next generation sequencing	Sampled pre-hospitalization and post-rehabilitation	Cloaca
Price et al. 2017	*Chelonia mydas*	Wild caught	Next generation sequencing	Sampled at capture	Cloaca

Table 1.2 old and new phyla names. In 2021 the international committee on Systematics of Prokaryotes changed the taxonomic names of 42 prokaryote phyla to align with the International Code of Nomenclature of Prokaryotes. Above is the list of the previously used and the new nomenclature. The old names are used for the phyla within this paper.

Old name	**New name**
Proteobacteria	Pseudomonadota
Firmicutes	Bacillota
Actinobacteria	Actinomycetota
Bacteroidetes	Bacteroidota
Fusobacteria	Fusobacteriota
Spirochaetes	Spirochaetota

CHAPTER II
REFERENCE INTERVALS FOR BLOOD ANALYTES IN LEATHERBACK NEONATES AND THEIR RELATION TO INCREASED INCUBATION TEMPERATUERS.

Abstract

There are seven extant species of sea turtles, all of which are imperiled. As such, large efforts are aimed at understanding the effects of climate change on these animals to aid conservation efforts. Currently, a large knowledge gap exists across neonatal leatherback baseline blood analytes and the potential impact of elevated incubation temperatures on health. Filling these knowledge gaps is critical for these imperiled species. All leatherback populations are considered endangered, but isolated populations of leatherbacks are at higher risk of extinction. I established reference intervals for key blood analytes. Additionally, to further understand the short-term and long-term impacts of increasing incubation temperatures, I compared blood analytes in neonates incubated in cooler nests to those that incubated in hot nests. I found that neonates from hot nests had higher packed cell volume (PCV), estimated total protein (TP), pre-albumin, and alpha-1 globulins at emergence. Additionally, at 3–4 weeks of age after captive-rearing, neonates from hot nests also had increased TP, albumin, alpha-1 globulins, and alpha-2 globulins compared to those that emerged from cooler nests. These alterations suggest that increased incubation temperatures are physiologically straining leatherback neonates even after emergence. These data may aid in explaining the decrease in survival seen in neonates incubated at higher temperatures and can be used to develop management strategies to mitigate the impacts of rising beach temperatures on leatherback neonates.

Introduction

Protein electrophoresis is a useful diagnostic tool in veterinary medicine, and its use has been expanding with our growing knowledge of its possible applications (Cray and Tatum 1998; Zaias and Cray 2002; Melillo 2013; Cray 2021). This technique relies upon the separation of protein molecules in the blood across an electrical field (Eckersall 2008; Cray 2021). The proteins are separated by charge and size and then categorized into fractions (Eckersall 2008; Cray 2021). The number of fractions can vary by species but generally include albumin, alpha globulins, beta globulins, and gamma globulins (Eckersall 2008; Cray 2021). Alterations in these fractions are then used to aid in the diagnosis of infectious diseases or inflammatory processes (Cray and Tatum 1998; Zaias and Cray 2002; Melillo 2013; Cray 2021). This diagnostic tool has become especially useful in non-traditional species (Zaias and Cray 2002; Melillo 2013; Cray 2021).

To date protein electrophoresis has been used in multiple species of sea turtles (Gicking et al., 2004; Deem et al., 2006; Innis et al., 2010; Osborne et al., 2010; Perrault et al., 2014; Flint et al., 2015; Stacy et al., 2019; Toonder et al., 2020). Values for protein electrophoresis have been published for free-ranging greens (*Chelonia mydas*) (Osborne et al., 2010; Flint et al., 2015; Toonder et al., 2020), loggerheads (*Caretta caretta*) (Gicking et al., 2004; Osborne et al., 2010;), and leatherbacks (*Dermochelys coriacea*) (Deem et al., 2006; Perrault et al., 2014; Stacy et al., 2019). However, most of the research to date has focused on establishing values in nesting females or in clinically healthy free-ranging immature sea turtles (Gicking et al., 2004; Deem et al., 2006; Osborne et al., 2010; Perrault et al., 2014; Flint et al., 2015; Stacy et al., 2019; Toonder et al., 2020). Establishing species and age class-specific blood analyte reference intervals (RI) is essential for effective application of reference intervals both clinically and for conservation

purposes (Stacy and Innis 2017; Stacy et al., 2017; Deem and Harris 2017). Thus, further investigations are necessary.

The leatherback sea turtle is the largest species of sea turtle and is taxonomically distinct from all other species of sea turtles (NMFS and USFWS, 2020). This taxonomic divergence brings with it both anatomical, physiologic, and behavioral characteristics that differentiate the leatherback from other sea turtle species (Boylan et al., 2017a,b; Hoopes et al., 2017). Currently, all leatherback populations are considered endangered, and some subpopulations are critically imperiled (NMFS and USFWS, 2020). As such, population recovery strongly depends on conservation efforts that are fueled by a better understanding of the species health (Deem et al., 2001; Jones et al., 2011). Likely due to the oceanic/pelagic lifestyle of the leatherback sea turtle, there is a lack of species and age class-specific reference intervals for these animals (Jones et al., 2011; Perrault et al., 2022). Due to its differences from other species, inferences on health and reference intervals made from studies in other species are likely unreliable when applied to leatherbacks.

Reference intervals in leatherback neonates are essential for health assessments (Stacy and Innis, 2017; Deem and Harris 2017). Beyond being used clinically to assess individual health, these baseline values can also be applied in a variety of ways to aid conservation efforts. One of the suggested approaches to aid in the recovery of leatherback populations is head-starting (Crespo-Picazo et al., 2017; Kanghae et al., 2024). However, baseline health information is limited for use in such programs and many health challenges are encountered during the captive rearing of leatherbacks (Jones et al., 2011; Kanghae et al., 2024). Baseline blood analytes could be used to assess health at entry to such facilities to promote positive outcomes. Furthermore, monitoring these values in individuals over time could aid in the improvement and continued development of ideal care and husbandry techniques. Improving outcomes in head-starting programs would significantly enhance our ability to support the recovery of imperiled leatherback populations.

In addition to head-starting, large conservation efforts are currently being aimed at understanding the impact of increasing incubation temperatures on neonates at emergence (Fleming et al., 2020; Page-Karjian et al., 2022; Perrault et al., 2022; Seaman and Milton 2023). Alterations in blood analytes correlated with increasing incubation temperatures can be used to inform critical conservation efforts and mitigation strategies (Deem et al., 2001; Stacy et al., 2017; Fleming et al., 2020; Perrault et al., 2022). Although leatherbacks may be difficult to study, fundamental data gaps urgently need filling to further our ability to preserve this endangered species. Therefore, the objectives of this study were to establish RIs for packed cell volume (PCV), estimated total protein (TP), and protein electrophoresis in leatherback neonates at two different life-stage classes (e.g., hatchling and post-hatchling) and assess the impacts of increasing incubation temperatures on these values.

Methods

Study animals and site

The leatherback neonates involved in this investigation originated from naturally oviposited nests on Juno Beach and Boca Raton, Florida. Neonates were collected during the 2021, 2022, and 2023 hatching seasons. All nests were fitted with HOBO® temperature data

loggers that recorded incubation temperature at 15- or 30-minute intervals. Throughout each season nests were routinely monitored for emergence. Upon emergence all neonates were transported to the Florida Atlantic University (FAU) Marine Laboratory for assessment and entrance into the research colony.

Neonate selection and husbandry

Within 12 hours of emergence all neonates were visually examined. Up to 5 neonates from each nest were selected for entrance into quarantine for the research colony at the FAU Marine Laboratory. All neonates selected for entrance into the colony appeared clinically normal (i.e., active, alert, and had no obvious external abnormalities). Neonates were housed and cared for as specified in Kuschke et al., 2023.

Husbandry

All neonates were maintained at the FAU Marine Laboratory for an on-going unrelated study. All tanks were indoors and equipped with an open-flow sea water system. Fluorescent lighting was provided using UVA-UVB light fixtures placed 45 cm above each tank. The lights were on a 12:12 hour cycle. Atlantic ocean water was pumped into the facility and filtered through two (100 μm) filtration socks, cleaned via protein skimming, and then treated with ultraviolet light. Optimal water temperatures (23–5°C) were maintained using a chiller. Neonates were fasted for the first 6 days to allow consumption of the yolk sac and then fed a gelatin-based diet 3 times a day at 1-2% of body mass each meal for a total consumption of 4-6% of body mass. After mid-day feedings turtles were given a fresh-water shower for 15–20 minutes to simulate mid-afternoon rainstorms in the area.

Sample collection

Blood samples were collected from neonates on the day of emergence and again at 3–4 weeks of age. Blood sampling followed the same procedure at both time points. After a single application of 0.05% chlorhexidine solution and 70% isopropyl alcohol, blood was collected from the external jugular vein using a heparinized 26-gauge needle. Blood collection did not exceed 0.7% of the total body mass (g) of the animal or 0.2 mL.

Sample processing

In all three years packed cell volume (PCV) was determined immediately following blood collection. A microhematocrit capillary tube was filled, centrifuged (13,000 g) for 1 minute, and a microhematocrit reader was used to measure the PCV. Prior to clotting, the remaining blood, was transferred into a 1.5 mL microcentrifuge tube and centrifuged at 6000 RPM for 1 minute. A small amount of plasma was used to estimate total protein (TP) via refractive index using a Fisherbrand™ handheld analog clinical refractometer with automatic temperature compensation. During the 2022 and 2023 seasons the remaining plasma was transferred to a 1.5 mL cryotube and stored at -80°C. Frozen plasma was then submitted to the Avian & Wildlife Laboratory (University of Miami, Miami, Florida) for analysis by agarose gel protein electrophoresis (AGE). The methods described by Cray (2021) were used to perform AGE.

Data analysis

Reference intervals for PCV and TP were established following the American Society of Veterinary Clinical Pathology RI guidelines using nonparametric methods using the Reference Interval package in RStudio (Finnegan, 2022; R Core Team, 2023). Due to sample size limitations, (i.e., $40 \leq x < 120$) reference intervals for all AGE values were calculated using robust methods (Friedrichs et al. 2012). Box-Cox transformations were employed when necessary. Outliers were detected using Horn's algorithm using Tukey's interquartile fences (Horn and Pesce 2003) and were subsequently removed. Incubation temperature data were converted into the percent of time spent at or above 31°C. This temperature cut off (31°C) was chosen because the average temperature across all nests was ~31°C. Then the percent of incubation time spent at or above 31°C was used to classify nest with incubation temperatures as either cooler or hot. Each neonate was treated independently and placed into one group based on this classification. Spending <40% of incubation at or above 31°C was considered cooler and spending ≥40% of incubation at or above 31°C was considered hot. This percentage (40%) was chosen as the cut off between groups as the average percent of incubation spent at or above 31°C was ~40%. Additionally, this cut off allowed for a fairly even number of nests to fall within each group. Due to not all bloody analytes having normal distribution, a Wilcoxon Rank Sum test was used to compare blood analytes at both time points between the cooler and hot group.

Results

Study animals

From 2021 to 2023, a total of 177 leatherback neonates from 37 different nests were included in this study. All neonates appeared clinically normal at the time of sampling. Mean, median, range, and RIs for all blood analytes at emergence and 3-4 weeks of age (Mean 23.53±2.75 days of age) are reported in Tables 2.1 and 2.2.

Comparisons between hot and cooler incubation temperatures

At emergence PCV ($P = .024$; W = 2402.5), TP ($P = .029$; W = 949), pre-albumin ($P = .008$; W = 949), and alpha-1 globulins ($P < .001$; W = 785.5) were all significantly higher in neonates that emerged from hot nests (Figures 2.1-2.4). At 3-4 weeks of age TP ($P = 0.044$; W = 1425), albumin ($P = .021$; W = 583), alpha-1globulins ($P = .030$; W = 598.5), and alpha-2 globulins ($P < .001$; W = 419.5) were significantly higher in neonates that incubated in hot nests (Figures 2.5-2.8).

Discussion

Reference intervals

Species and age-class specific RIs in wildlife not only inform individual clinical care but can also be used to understand the impact of environmental changes overtime in both individuals and the population (Deem et al., 2001; Deem and Harris 2017; Stacy et al., 2017; Fleming et al., 2020; Perrault et al., 2022). This study was unique in comparison to other leatherback health studies as it established RIs in leatherback neonates at both emergence (i.e., the hatchling stage)

and at 3–4 weeks of age (i.e., post-hatchling stage). At emergence the observed PCV and TP were similar though slightly lower than previously reported values in leatherbacks (Table 2.3; Reina et al., 2002; Perrault et al., 2022). However, at 3–4 weeks of age, there was a decrease in both PCV (from 0.24±0.03 L/L to 0.19±0.05 L/L) and TP (from 32±6 g/L to 19±7). This finding was unexpected as blood analytes typically increase with somatic growth in sea turtles (Frair 1977; Stacy and Innis 2017; Stacy et al 2018; Perrault et al., 2020). However, previous studies investigating PCV and TP suggested that neonates are suffering from altered fluid dynamics at emergence which would then falsely increase both PCV and TP (Fleming et al., 2020; Perrault et al., 2022). The decrease in PCV and TP seen at 3–4 weeks of age may reflect more normal values with less influence from possible subclinical dehydration. Neonates at 3–4 weeks of age were maintained in life-stage class appropriate conditions including a fully aquatic environment, a consistent diet, and simulated freshwater showers daily. These conditions likely allowed the neonates sufficient physiologic support to correct any subclinical dehydration and then maintain appropriate osmoregulation in the blood. This is further supported as the values seen at 3–4 weeks of age were similar to those reported in other neonatal (post-hatchling) sea turtles (Rousselet et al., 2013; March et al., 2019). Lymph contamination is another possible cause of this decrease that cannot be ruled out, but it is unlikely as no visible contamination occurred in any of the samples included in the study.

The RIs for AGE herein were similar between the two time points (i.e., emergence and 3–4 weeks of age). However, albumin, alpha-1 globulins, and alpha-2 globulins decreased at 3–4 weeks of age in comparison to neonates at emergence. The decrease in albumin from 11.4±2 g/L to 5.8±2.1g/L at 3–4 weeks of age, like with PCV and TP, may be a product of falsely increased albumin at emergence due to subclinical dehydration (O'Connell et al., 2005; Stacy and Boylan 2014). Consequently, the measured albumin at 3-4 weeks of age may be the product of the value returning to a more normal level after rehydration. Following entrance into the FAU Marine Laboratory and being placed into tanks with ocean water, neonates likely underwent physiologic efforts aimed at restoring homeostasis and hydration status.

The observed changes in alpha-1 and alpha-2 globulins may also be following a similar trend of returning to more normal levels at 3–4 weeks of age. Alpha-1 globulins dropped from 4.3±1.2 g/L to 2.1±1.2 g/L at 3–4 weeks of age and alpha-2 globulins dropped from 5.8±1.7 to 3.6±1.7 g/L at 3–4 weeks of age. Recent studies have found evidence of possible inflammation in sea turtle neonates at emergence correlated with increased incubation temperatures (Fleming et al., 2020; Perrault et al., 2022). Both alpha-1 and alpha-2 globulins are composed of acute phase proteins (APPs) which increase with acute inflammation (O'connell et al., 2005). At emergence, both values may have increased due to acute inflammation, as previously reported by Perrault et al., 2022. Then 3–4 weeks later the inflammation decreases, and both values decrease. The observed decrease could be attributed to ideal husbandry and nutritional conditions at the FAU Marine Laboratory, removal from the high heat nest environment, or other unknown variables.

Current studies in protein electrophoresis in leatherbacks are limited to nesting females (Deem et al., 2006; Perrault et al., 2012, 2014; Stacy et al., 2019). When comparing the RIs reported here to those in nesting females, most concentrations were similar except for albumin and alpha-1 globulins (Table 2.3). Albumin concentrations at both time points (emergence and 3–4 weeks) appears to be lower than previous reported concentrations in nesting females (Deem

et al., 2006; Perrault et al., 2014; Stacy et al., 2019). However, this is expected as nesting females are known to have higher than normal albumin levels due to reproductive demands, though this value has been reported to decrease through the nesting season secondary to fasting (Deem et al., 2009; Perrault et al., 2014; Stacy and Boylan 2014). Additionally, increases in albumin concentrations have also been positively correlated with size (Stacy et al., 2018; Perrault et al., 2020). As such, it would be expected that albumin concentrations would be lower at emergence and 3–4 weeks when compared to larger leatherbacks. The ranges of alpha-1 globulin concentrations reported here are higher than those reported by Stacy et al., 2019 and Deem et al., 2006 in nesting females. While this was not expected because of their age and likely underdeveloped immune systems, it is possible that inflammation in sea turtle neonates (Fleming et al., 2020; Perrault et al., 2022) related to environmental stress such as increased incubation temperatures is leading to this increase.

Relationships to incubation temperature at emergence

When comparing blood analytes at emergence between the two temperature groups, trends similar to those previously reported in leatherback, loggerhead, and green turtles at emergence were observed (Fleming et al., 2020; Perrault et al., 2022; Page-Kargian et al., 2022). Neonates that emerged from hot nests had significantly increased PCV, TP, pre-albumin, and alpha-1 globulins. Increases in PCV and TP are likely due to subclinical dehydration caused by increased temperatures within the nest (O'connell et al., 2005; Stacy and Boylan 2014). Pre-albumin concentrations are generally very low in sea turtles and the significance of this increase is unknown (Gicking et al., 2004; Deem et al., 2006; Deem et al., 2009). The function of pre-albumin in sea turtles is currently unknown and warrants further investigation though, it may serve a similar function as in humans and act as a transport protein (Melillo, 2013).

The alpha-1 globulin fraction is primarily composed of acute phase proteins. Acute phase proteins are proteins within the blood that are created and released in response to infection, inflammation, trauma, or stress (Murata et al., 2004; Eckersall et al., 2008; Osborne et al., 2010; Cray, 2012). The increase in alpha-1 globulins in neonates emerging from hot nests suggests that increased incubation temperatures may cause inflammation or physiologic stress in leatherbacks at emergence. This finding is consistent with a study in chickens that found increased thermal stress increased acute phase protein concentrations (Hanafi 2022). Additionally, the positive correlation between temperature and alpha-1 globulins is also consistent with findings in other turtle species (Osborne et al., 2010; Andersson et al., 2021).

Relationships to incubation temperature in 3–4 week-old leatherback neonates

This study was the first to assess the potential longer-term impacts of increased incubation temperature on leatherback neonates. Alterations in blood analytes were observed between neonates that emerged from cooler and hot nests at 3–4 weeks of age. Total protein, albumin, alpha-1 globulins, and alpha-2 globulins were significantly higher in leatherback neonates that emerged from hot nests. These findings were surprising as a return to homeostasis was anticipated after 3–4 weeks. While it is possible that stress related to captivity may be preventing normalization of these values, it is likely not the only driving force behind these elevations. The higher TP observed may be due to continued mild/subclinical dehydration. Though this is unlikely as, physiologically, sea turtles exhibit efficient osmoregulation when

submerged in water (Ortiz et al., 2000; Innis et al., 2017). Juvenile sea turtles have shown the ability to return to homeostasis 48 hours after being returned to salt water (Innis et al., 2017). Therefore, the increase in TP is more likely due to an increase in the components that make up this blood analyte (O'connell et al., 2005; Eckersall et al., 2008; Stacy and Boylan 2014). Total protein is a measure of all proteins within the blood, which includes pre-albumin/albumin and globulins (e.g., alpha, beta, and gamma). Thus, the observed increase in albumin, alpha-1 globulins, and alpha-2 globulins likely drove the observed increase in TP.

The increase in albumin observed in turtles that emerged from hot nests was unexpected as albumin is considered a negative acute phase protein (Cray, 2012). Consequently, albumin should have been decreased in neonates that emerged from hot nests. Albumin is the most abundant plasma protein in the blood and is responsible for maintaining oncotic pressure within the body (Fanali et al., 2012). As such, when there is a decrease in blood volume (i.e., dehydration), it causes a false increase in measured albumin (Stacy and Boylan 2014). The increase in albumin seen at 3–4 week of age in neonates emerging from hot nests could potentially be due to continued mild/subclinical dehydration. However, as previously stated, this is unlikely because the turtles are housed in sea water and should not be struggling with osmoregulation (Ortiz et al., 2000; Innis et al., 2017). There are few other known significant causes of increased albumin in turtles but a positive relationship between albumin concentrations and size has been previously reported (Stacy et al., 2018; Perrault et al., 2020). However, mass has been negatively correlated to clutch deposition date in leatherback hatchlings, which is a proxy for incubation temperature (Perrault et al., 2021). This would suggest that turtles emerging from hot nests would be smaller and therefore have a lower albumin concentration. Unfortunately, comparisons of size or mass between groups were not explored in this study and should be considered in future work. Albumin concentrations are also known to increase in foraging leatherbacks (i.e., with eating) and decrease in nesting females through the nesting season due to fasting. It is possible that alterations in eating habits are resulting in the changes in albumin concentration between these groups. All the animals included in this study were fed 3 times a day to a total of 4-6% of body weight daily. However, differences in daily intake were not compared between groups and should be considered in the future.

The continued increase in alpha-1 and the new increase in alpha-2 globulins was surprising but not unreasonable considering previous research. Evidence of inflammation has previously been detected in sea turtle neonates emerging from hot nests (Fleming et al., 2020; Page-Karjian et al., 2022; Perrault et al., 2022), but this is the first record of possible inflammation persisting in neonates after emerging from hot nests. Continued inflammation in neonates emerging from hot nests suggests that increasing temperatures potentially have longer-term impacts on neonatal health after their emergence from nests. Consequently, the physiologic challenges facing neonates emerging from hot nests are likely negatively impacting their overall survival. Additional studies utilizing other measures such as white blood cell counts and other acute phase proteins should be explored to further our understanding of the possible inflammation and its physiologic impact on the neonates.

Conclusion

The results of this work provide RIs for several previously unstudied blood analytes in leatherbacks at emergence and 3–4 weeks of age. These data will be useful both on the

individual and population level. The species and age-class specific RIs provided herein will not only inform clinical care but can also be used to improve our ability to head-start leatherbacks and begin to understand the impacts of rising incubation temperatures on neonatal health and long-term survival. This study found that increasing incubation temperatures are correlated with significant alterations in blood analytes that persist for up to 3–4 weeks. The possible suboptimal physiologic states (i.e., subclinical dehydration and possible inflammation) observed at emergence and again at 3–4 weeks of age could potentially negatively impact overall fitness and survival of leatherback neonates after emergence from the nest. Accordingly, these data provide initial information to explain a cause for decreased survival in neonates incubating at higher temperatures. Furthermore, these findings can be useful for developing management strategies to mitigate the effects of elevated incubation temperatures on leatherback sea turtles.

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Appendix

Table 2.1 Measures of central tendency and reference intervals for packed cell volume, estimated total protein, and agarose gel protein electrophoresis in leatherback neonates at emergence. Nonparametric methods were used for sample sizes ≥120 and robust methods were used for sample sizes <120. Outliers were detected using Horn's algorithm using Tukey's interquartile fences and were subsequently removed. Box-Cox transformations were employed when necessary. *SD: standard deviation, RI: reference interval, CI: confidence interval, LRL: lower reference limit, URL: upper reference limit*.

Measurement/analyte	**Mean ± SD**	**Median**	**Range**	**N**	**RI**	**LRL 90% CI**	**URL 90% CI**
Packed cell volume (L/L)	0.24±0.03	0.24	0.16–0.31	156	0.17–0.30	0.16–0.18	0.29–0.31
Estimated total protein (g/L)	32±6	32	12–46	159	20–44	12–20	42–46
Albumin:globulin	0.59±0.09	0.60	0.35–0.78	104	0.42–0.77	0.39–0.44	0.75–0.80
Pre-Albumin (g/L)	0.3±0.4	0.2	0–2.5	104	0–1.7	0	1.0–1.9
Albumin (g/L)	11.4±2	11.6	7.4–16.0	99	7.5–15.5	6.9–8.1	15.1-16.1
Alpha-1 globulins (g/L)	4.3±1.2	4.4	1.4-7.4	102	1.9–6.8	1.5–2.3	6.5–7.1
Alpha-2 globulins (g/L)	5.8±1.7	5.6	2.2–10.6	102	2.2–9.1	1.6–2.6	8.5–9.6
Beta globulins (g/L)	6.3±1.7	6.2	2.8–10.3	99	2.6–9.6	2.1–3.1	9.1–10.1
Gamma globulins (g/L)	3.7±0.8	3.7	1.8–5.4	99	2.1–5.3	1.9–2.3	5.1–5.6

Table 2.2 Measures of central tendency and reference intervals for packed cell volume, estimated total protein, and agarose gel protein electrophoresis in leatherback neonates at 3-4 weeks of age. Nonparametric methods were used for sample sizes ≥120 and robust methods were used for sample sizes <120. Outliers were detected using Horn's algorithm using Tukey's interquartile fences and were subsequently removed. Box-Cox transformations were employed when necessary. *SD: standard deviation, RI: reference interval, CI: confidence interval, LRL: lower reference limit, URL: upper reference limit.*

Measurement/analyte	**Mean ± SD**	**Median**	**Range**	**N**	**RI**	**LRL 90% CI**	**URL 90% CI**
Packed cell volume (L/L)	0.19±0.05	0.19	0.10–0.29	122	0.10–0.28	0.10–0.12	0.26–0.29
Estimated total protein (g/L)	19±7	18	10–44	133	10–37	10–10	32–44
Albumin:globulin	0.45±0.10	0.44	0.25–0.75	80	0.24–0.64	0.21–0.27	0.60–0.67
Pre-Albumin (g/L)	0.2±0.3	0.1	0–1.7	83	0–0.7	0	0.5–0.8
Albumin (g/L)	5.8±2.1	5.1	2.4–12	83	1.2–10.2	0.4–1.7	9.6–11.2
Alpha-1 globulins (g/L)	2.1±1.2	1.7	0.4–5.4	83	0.4–5.0	0.3–0.6	4.4–6.4
Alpha-2 globulins (g/L)	3.6±1.7	3.3	0.9–7.9	83	1.1–8.3	0.8–1.3	7.0–9.4
Beta globulins (g/L)	5.6±2.8	4.6	2.2–14.5	82	2.2–13.3	2.1–2.5	11.1–17.6
Gamma globulins (g/L)	2.3±0.8	2.2	1.0–4.7	73	0.5–3.8	0.1–0.7	3.5–4.1

Table 2.3 Comparison of mean values from the current study to previous studies measuring packed cell volume, estimated total protein, and agarose gel protein electrophoresis in leatherbacks. *SD* standard deviation

Measurement/analyte	**Mean ± SD** **Emergence**	**Mean ± SD** **3–4 weeks of age**	**Mean ± SD** **Perrault et al., 2021**	**Mean ± SD** **Stacy et al., 2019**
Packed cell volume (L/L)	0.24±0.03	0.19±0.05	0.30±0.04	0.36±0.01
Estimated total protein (g/L)	32±6	19±7	41±7	37±5
Albumin:globulin	0.59±0.09	0.45±0.10	NA	0.43±0.07
Pre-Albumin (g/L)	0.3±0.4	0.2±0.3	NA	0.31±0.25
Albumin (g/L)	11.4±2	5.8±2.1	NA	10.62±1.90
Alpha-1 globulins (g/L)	4.3±1.2	2.1±1.2	NA	1.80±0.30
Alpha-2 globulins (g/L)	5.8±1.7	3.6±1.7	NA	6.59±1.35
Beta globulins (g/L)	6.3±1.7	5.6±2.8	NA	10.39±2.00
Gamma globulins (g/L)	3.7±0.8	2.3±0.8	NA	6.84±1.16

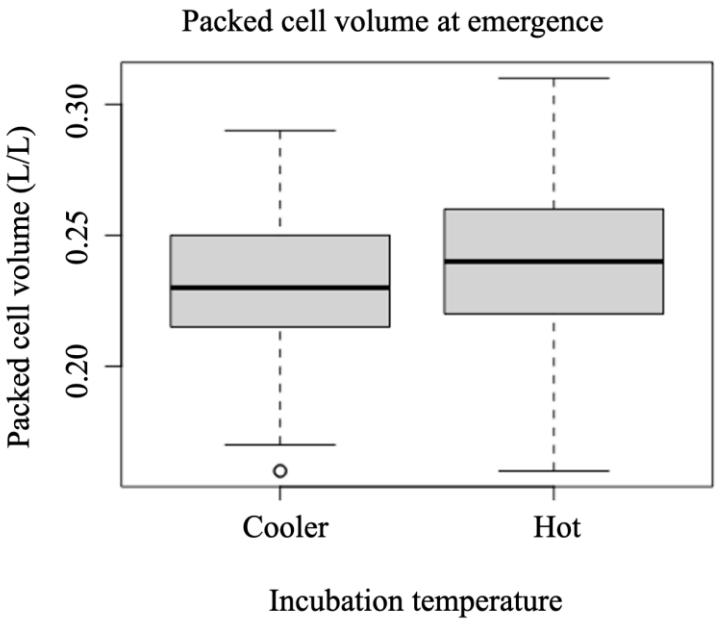


Figure 2.1 Boxplot of packed cell volume (PCV) at emergence comparing values between neonates that incubated in cooler versus nests. PCV was significantly higher in neonates that incubated in hot nests (P = .024; W = 2402.5). *Gray boxes are the first and third quartiles; thick black lines designate the median value; dashed lines are the minimum and maximum values; open circles are outliers; Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

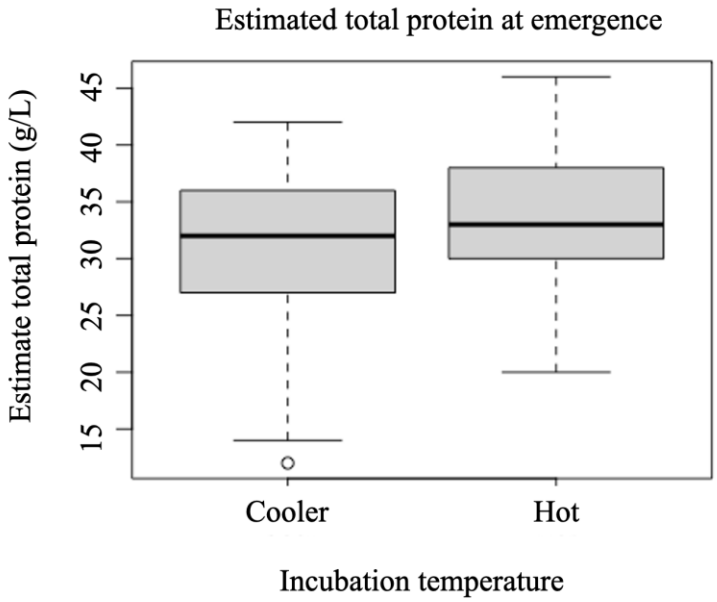


Figure 2.2 Boxplot of estimated total protein (TP) at emergence comparing values between neonates that incubated in cooler versus hot nests. TP was significantly higher in neonates that incubated in hot nests (P = .029; W = 949). *Box-plot is formatted as in Figure 2.1; Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥ 31 °C*

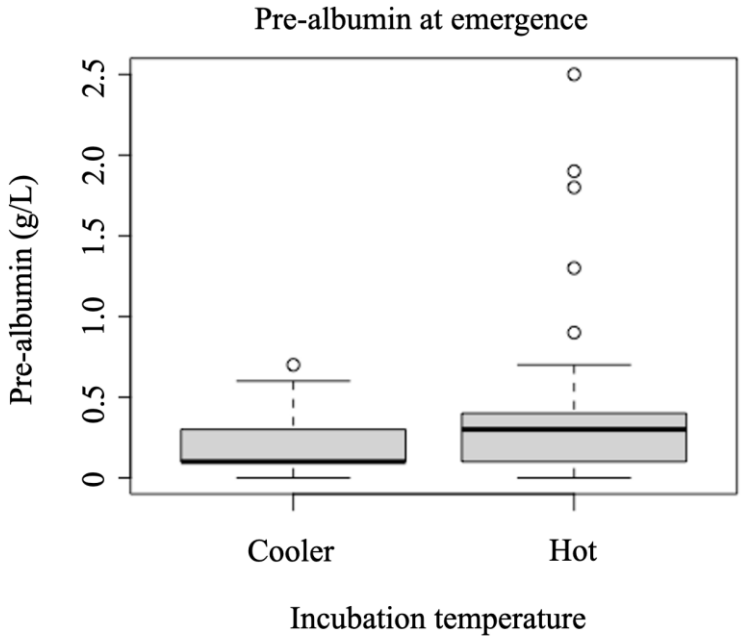


Figure 2.3 Boxplot of pre-albumin at emergence comparing values between neonates that incubated in cooler versus hot nests. Pre-albumin was significantly higher in neonates that incubated in hot nests (P = 0.008; W = 949). *Box-plot is formatted as in Figure 2.1; Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

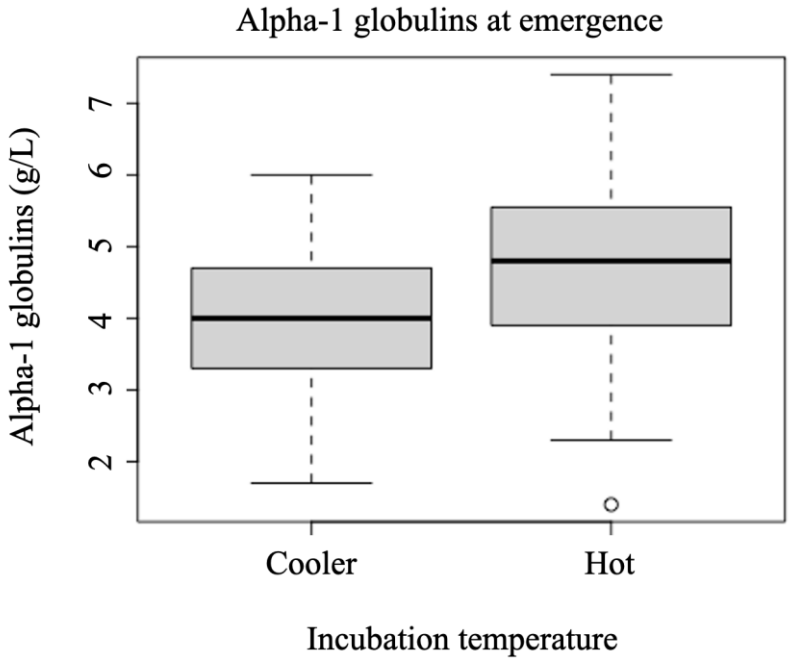


Figure 2.4 Boxplot of alpha-1 globulins at emergence comparing values between neonates that incubated in cooler versus hot nests. Alpha-1 globulins was significantly higher in neonates that incubated in hot nests ($P < .001$; $W = 785.5$). *Box-plot is formatted as in Figure 2.1; Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

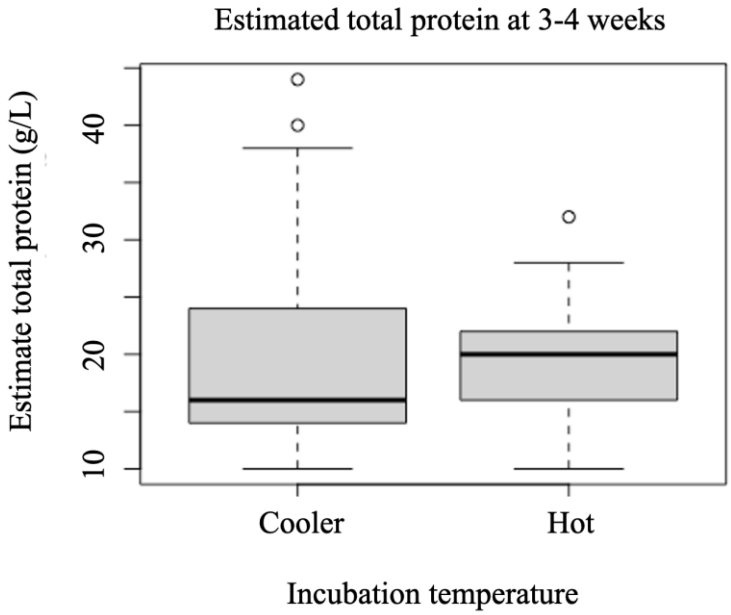


Figure 2.5 Boxplot of estimated total protein (TP) at 3–4 weeks of age comparing values between neonates that incubated in cooler versus hot nests. TP was significantly higher in neonates that incubated in hot nests (P = 0.044; W = 1425). *Box-plot is formatted as in Figure 2.1; Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

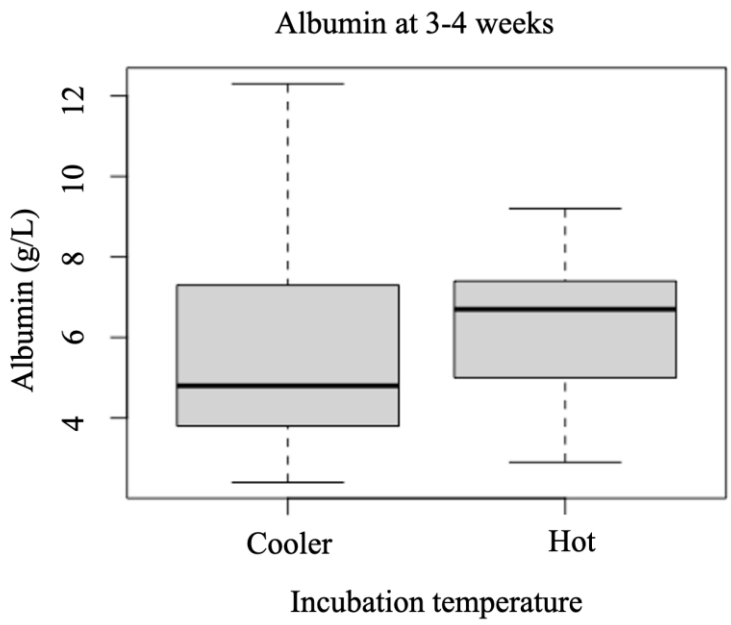


Figure 2.6 Boxplot of albumin at 3–4 weeks of age comparing values between neonates that incubated in cooler versus hot nests. Albumin was significantly higher in neonates that incubated in hot nests (P = .021; W = 583). *Box-plot is formatted as in Figure 2.1; Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

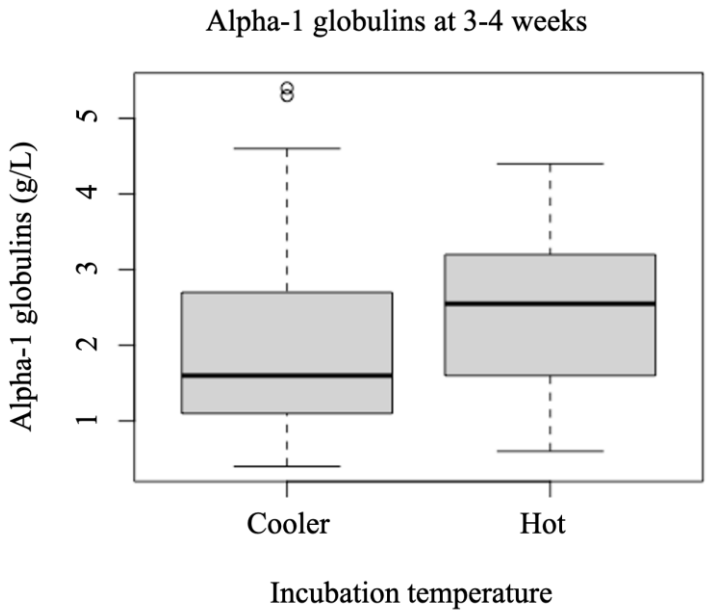


Figure 2.7 Boxplot of alpha-1 globulins at 3–4 weeks comparing values between neonates that incubated in cooler versus hot nests. Alpha-1 globulins were significantly higher in neonates that incubated in hot nests (P = .030; W = 598.5). *Box-plot is formatted as in Figure 2.1; Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

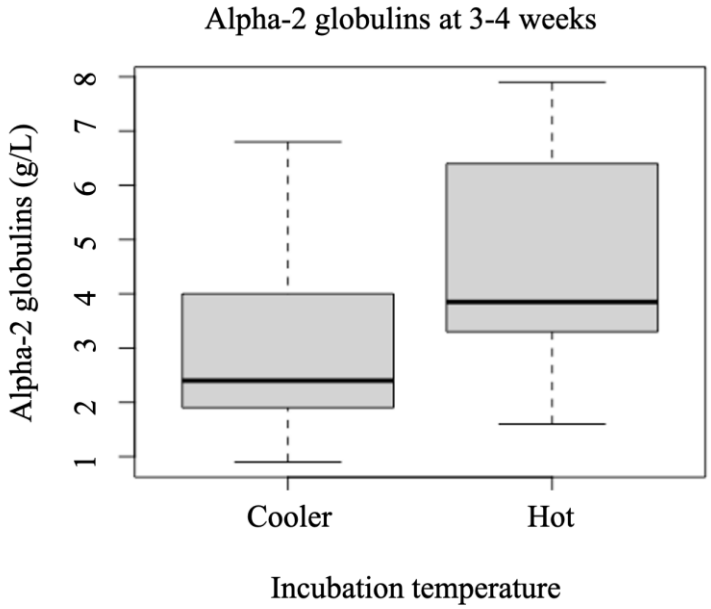


Figure 2.8 Boxplot of alpha-2 globulins at 3–4 weeks comparing values between neonates that incubated in cooler versus hot nests. Alpha-2 globulins were significantly higher in neonates that incubated in hot nests ($P < .001$; $W = 419.5$). *Box-plot is formatted as in Figure 2.1; Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

CHAPTER III
BASELINE SKIN MICROBIOTA OF THE LEATHERBACK SEA TURTLE

Abstract

The integumentary system of the leatherback sea turtle (*Dermochelys coriacea*) is the most visible and defining difference of the species, with its smooth and waxy carapace and finely scaled skin, distinguishing it from the other six sea turtle species. The skin is the body's largest organ and serves as a primary defense against the outside world and is thus essential to health. To date, we have begun to understand that the microorganisms located on the skin aid in these functions. However, many host-microbial interactions are not yet fully defined or understood. Prior to uncovering these crucial host-microbial interactions, we must first understand the communities of microorganisms present and how they differ through life-stage classes and across the body. Here, we present a comprehensive bacterial microbial profile on the skin of leatherbacks. Using next generation sequencing (NGS), we identified the major groups of bacteria on the skin of neonates at emergence, neonates at 3–4 weeks of age (i.e., post-hatchlings), and nesting females. These data show that the predominant bacteria on the skin of the leatherback are different at each life-stage class sampled. This suggests that there is a shift in the microbial communities of the skin associated with life-stage class or even possibly age. We also found that different sample locations on the nesting female (i.e., carapace and front appendages) have significantly different communities of bacteria present. This is likely due to differences in the microhabitats of these anatomic locations and future studies should explore if this variation also holds true for neonates. These data define baseline skin microbiota on the leatherback and can serve as a foundation for additional work to broaden our understanding of the leatherbacks' host-microbial interactions, the impacts of environmental changes or stressors over time, and even pathogenicity of disease processes.

Introduction

The leatherback sea turtle (*Dermochelys coriacea*) is the largest species of sea turtle and the sole extant genus of the family Dermochelyidae (Boylan et al., 2017). One notable difference from other marine turtles is the leatherback integumentary system. In contrast to scalation and heavy cornification of "hard-shelled" sea turtles, adult leatherback integument is pliable, and waxy (Boylan et al., 2017). Neonatal leatherback skin is uniquely composed of small domed scales (Boylan et al., 2017). Leatherback integument is not only grossly different from that of other marine turtles but is also different in composition. The outer most layer of skin (stratum corneum) is composed mainly of resilient and flexible alpha-keratin underlying a thin layer of (tough) beta-keratin (Boylan et al., 2017). Other marine turtle species' stratum corneum is composed of a thick layer of beta-keratin (Boylan et al., 2017).

The integumentary system in leatherbacks functions in thermoregulation, osmotic balance, prevention of dehydration, camouflage, synthesis of vitamin D precursors, and as a barrier to infection and harmful wavelengths of light (Boylan et al., 2017). The skin also is host to a complex and dynamic community of microorganisms that together make up the skin microbiota. In recent years, the skin microbiota has been a major focus of research in humans and animals (Ursell et al., 2012; Apprill, 2017; Ross et al., 2018; Ross et al., 2019; Robollar et al., 2020; Kuschke, 2022). This surge in research stems from the growing understanding that microbiota, both skin and gut, can significantly impact host functions such as development, behavior, and health (Sommer and Backhed 2013; Selosse et al., 2014; Colston and Jackson 2016; Walke and Belden 2016; Simon et al., 2019). More specifically, skin microbiota are the

primary defenses against invading pathogens and are thought to play significant roles in host health, immune function, host resistance, and responses to endogenous and exogenous stressors (Harris et al., 2009; Selosse et al., 2014; Walke et al., 2015; Colston and Jackson 2016; Walke and Belden 2016; Jiménez and Sommer 2016; Kho and Lal 2018; Simon et al., 2019).

Research exploring the skin microbiota of marine animals has become an area of great interest because of the skin's constant contact with seawater and the microorganism that live within it (Bolten and Bjorndal 1992; Apprill et al., 2011; Bik et al., 2016; Chiarello et al., 2017; Russo et al., 2018;). Investigations of marine mammals have begun to identify microbes that make up species-specific core microbiota (Apprill et al., 2011; Bik et al., 2016; Russo et al., 2018; Chiarello et al., 2017). Unfortunately, few studies have investigated skin microbiota in reptiles (Colston and Jackson 2016; Ross et al., 2019; Alduina et al., 2020; Parks et al., 2020; McKnight et al., 2020). Moreover, the information on the bacterial microbiota of free-ranging sea turtles is limited, and no integumentary flora studies have utilized contemporary culture-independent methods (Colston and Jackson 2016; Kuschke, 2022). The reason for this data gap is likely multifactorial. In the case of leatherbacks, it is likely due to the unique challenges they pose to researchers both because of their oceanic-pelagic lifestyle and the difficulty of maintaining them (at any life-stage class) in human care for research or rehabilitation (Jones et al., 2000; Jones et al., 2011). However, investigations into the skin microbiota of leatherbacks can provide essential insight for conservationists, researchers, and rehabbers on their health (Kuschke, 2022). Additionally, application of these data will broaden our understanding of the leatherbacks' host-microbial interactions, the impacts of environmental changes or stressors over time, and even pathogenicity of disease processes (Colston and Jackson 2016; Kuschke, 2022; Kuschke et al., 2023).

Prior to exploring host-microbial interactions, microbial baselines must be obtained. A robust survey of baseline skin microbiota in clinically healthy animals is necessary before exploration of its connection to health, disease, and environmental variables can be explored. Once baselines are obtained, research into the skin microbiota's role in health, immune function, resistance to endogenous stressors, and disease can commence. In this study, I used next-generation sequencing (NGS) to identify and characterize the skin microbiota of leatherback sea turtles at different life-stage classes (neonates and nesting females).

Methods

Neonate selection and husbandry

The leatherback sea turtle neonates involved in this study originated from naturally oviposited nests on Juno Beach and Boca Raton, Florida. Neonates were collected during the 2021, 2022, and 2023 hatching seasons (May–August). Throughout each season, nests were routinely monitored for emergence. Upon emergence all neonates were transported to the Florida Atlantic University (FAU) Marine Laboratory in Boca Raton, Florida for assessment and entrance into the research colony. All neonates were visually examined within 12 hours of emergence. Up to 5 neonates from each nest were selected for entrance into the research colony at the FAU Marine Laboratory. All of those selected for entrance into the colony appeared clinically normal (i.e., active, alert, and had no obvious external abnormalities). All neonates were housed indoors at the FAU Marine Laboratory. Atlantic ocean water was pumped in

through a near shore collection and was filtered through two (100 μm) filtration socks, cleaned via protein skimming, treated with ultraviolet light sufficient to eliminate microbes (Kuschke et al., 2023). Water was also sent through a chiller to maintain appropriate temperatures for leatherback sea turtles (23-25°C) **(**Kuschke et al., 2023). Fluorescent lights (UVA-UVB) on a 12:12 hour cycle were hung 45 cm above the tanks **(**Kuschke et al., 2023).

Neonate sample collection

Skin swabs were collected from neonates entering the FAU research colony on the day of emergence and again at 3-4 weeks of age (19-30 days). Sample collection followed the same procedure at both time points. Each neonate was rinsed with 50 mL of sterile nano-pure water four times. Due to their small size, a single sterile swab (rayon or polyester) was used to sample the animal's whole integument including the head, carapace, plastron, and all four limbs. Swabbing was repeated in duplicate for each neonate. Each swab was placed into a sterile vial and was stored at -80°C until DNA extraction.

Nesting female selection and sample collection

All nesting females were sampled from Juno and Jupiter Beach, Florida. Samples were collected during the 2022 and 2023 nesting seasons. For each nesting female, sample collection began after initiation of egg deposition and the nesting fixed action patter, during which the nesting female is nonresponsive to manipulation **(**Dutton and Dutton 1994**)**. Prior to sampling, a ~30 cm area on the cranial half of the carapace was rinsed with 500mL of sterile nano-pure twice. Then using sterile swabs (rayon or polyester) a ~10 cm by 10 cm area in the rinsed region was swabbed twice. The rinsing and swabbing process was then repeated on the proximal region of the flipper (left or right as available due to positioning). Samples were placed into individual sterile tubes and then on ice until they could be stored in a freezer at -80°C until DNA extraction.

DNA extractions

All DNA extractions were performed using the DNeasy® PowerSoil® Pro Kit (QIAGEN®) according to manufacturer's protocol with the following modifications. Swabs were placed into the PowerBead Pro Tubes with 800 μl of CD1 solution and left to sit for 5 minutes. PowerBead Pro Tubes were then vortexed for 10 minutes with the swabs still in the tubes. The supernatant was removed from the PowerBead Pro Tube from around the swab (i.e., the swab was still within the tube). The manufacturer's protocol was then followed until the final step which had the following modification. Solution C6 was left to sit on the center of the white filter membrane for 5 minutes prior to centrifuging. All DNA was stored at -80°C until sequencing.

Next-generation sequencing

All DNA was submitted to the University of Tennessee Genomics Core for polymerase chain reaction (PCR) and NGS. Next-generation sequencing was performed using the V3-V4 region of the 16S rRNA gene with forward primer 5' CCTACGGGNGGCWGCAG 3' and reverse 5' GACTACHVGGGTATCTAATCC 3' on the Illumina MiSeq (Fadrosh et al., 2014).

Data Processing

Sequence data were analyzed using the DADA2 pipeline (v1. 18.0; Callahan et al., 2016; Bailly et al., 2022) in RStudio (v4.3.1; R Core Team 2023). Taxonomy was assigned using the IDTAXA algorithm in the DECIPHER package (Wright 2016; Murali et al., 2018). Samples with <10,000 reads were removed prior to statistical analysis.

Statistical analysis

All data analysis was performed using Rstudio (v4.3.1; R Core Team 2023). Packages used included 'phyloseq', 'vegan', 'devtools', 'microbiome', and 'ggplot' (McMurdie and Holmes 2023; Oksanen et al., 2022; Wickham et al., 2022; Lahti and Shetty 2019; Wickham 2016). Alpha diversity was explored using Observed OTUs, Shannon index, Chao1 estimates, and Inverse Simpson index. Alpha diversity comparisons between age-classes and between sample location on nesting females were made using a pairwise Wilcoxon rank sum test with Holm p-values that were adjusted for multiple comparisons (Scheelings et al., 2020a). Beta diversity was explored using principal coordinate analysis (PCoA) (Bray-Curtis) and pairwiseAdonis tests were used to compare between life-stage classes and between sample location on nesting females (Scheelings et al., 2020a).

Results

Comparisons across life stages

From 2021 to 2023 a total of 178 neonatal leatherbacks from 38 nests were sampled at emergence and 140 of those turtles were also sampled again at 3-4 weeks of age (Table 3.1). During the 2022 and 2023 nesting seasons a total of 37 nesting females were sampled. Across all life-stage classes, there were 5 phyla that were predominant: Proteobateria, Bacteroidota, Patescibacteria, Bdellovibrionota and Firmicutes (Table 3.2, Figure 3.1). Relative abundance of each phylum was similar across all leatherbacks except for Patescibacteria, which was found in higher abundance in nesting females than in neonates at emergence or neonates at 3–4 weeks of age. There were 6 prominent families across all leatherbacks that included: Flavobacteriacea, Rodobacteraceae, Xanthomonadaceae, Moraxellaceae, Saprospiraceae, and Pseudomonaadaceae (Table 3.3, Figure 3.2). At emergence, the most abundant families were Xanthomonadaceae and Moraxellaceae. However, at 3–4 weeks, a shift in the most abundant families to Flavobacteriacea and Rodobacteraceae was observed. Flavobacteriaceae and Rodobacteraceae were also the most abundant bacterial families found on the nesting females.

Analysis of alpha diversity revealed significant differences between all life-stage classes in Observed Operational Taxonomic Units (OTUs) (df = 2, Table 3.4 Figure 3.3) and in species richness between samples as measured by Chao1 estimates (df = 2, Table 3.5; Figure 3.4), Shannon (df = 2, Table 3.6; Figure 3.5), and Inverse Simpson indices (df = 2, Table 3.7; Figure 3.6). For beta diversity I detected clustering patterns for all life-stage classes of leatherbacks sampled (i.e., emergence, 3–4 weeks, and nesting females). Significant differences in microbial communities existed between all three life-stage classes ($R^2 = 0.16$, Table 3.8; Figure 3.7).

Comparisons between carapace and flipper on nesting females

Predominant phyla on both the carapace and flipper of nesting females were similar. However, Cyanobacteria and Verrucomicrobiota were both more predominant on the flipper while, Patescibacteria was found in higher abundance on the carapace (Table 3.9, Figure 3.8). Bacterial families were also very similar between sample locations on the nesting females except Rubritaleaceae was notably more abundant on the front flipper (Table 3.10, Figure 3.9). Significant differences in alpha diversity were seen in Observed OTUs (df = 1, $p = 0.004$; Figure 3.10) and in species richness between carapace and flipper skin as measured by Chao1 estimates (df = 1, $p = 0.004$; Figure 3.11), Shannon (df = 1, $p < 0.001$; Figure 3.12), and Inverse Simpson indices (df = 1, $p < 0.001$; Figure 3.13). Analysis of beta diversity also revealed significant differences in microbial communities between the two sample locations (i.e., carapace and front flipper) ($R^2 = 0.09$, $p = 0.001$; Figure 3.14).

Discussion

In this study, I provide essential baseline microbiota data in leatherbacks and show that the composition of skin microbiota changes between life-stage classes and location on the body. These data fill a key knowledge gap for this species and can be used as a tool to assess population health and understand the impacts of environmental shifts (Kuschke, 2022). Due to their long oceanic-pelagic life history, sea turtles are often referred to as sentinel species (Aguirre and Lutz 2004; Hazen et al., 2019; Drane et al., 2021). As such, they are used to assess environmental changes and their potential impacts on marine life (Aguirre and Lutz 2004; Hazen et al., 2019; Drane et al., 2021). Due to the skin microbiota's constant contact with seawater, it is an ideal location to assess the impacts of environmental factors on health (Apprill et al., 2011; Bik et al., 2016; Chiarello et al., 2017; Russo et al., 2018). Using these baseline data, researchers and conservationists can now begin to investigate host-microbial interactions, the impacts of environmental changes or stressors over time, and even extrapolate pathogenicity of skin disease (Colston and Jackson 2016; Kuschke, 2022; Kuschke et al., 2023).

Comparisons across life stages

Across all life-stage classes, relative abundance was similar for the top 2 most predominant phyla (i.e., Proteobacteria and Bacteroidota). These results are similar to those reported in Mediterranean loggerheads (Kanjer et al., 2022). However, the third most predominant bacterial phylum in leatherbacks differs from that reported in loggerheads (i.e., Bdellovibrionota). In neonates at emergence and 3–4 weeks of age the third most predominant bacterial phylum was Actinobacteriota and in nesting females it was Patescibacteria. Patescibacteria was found in higher abundance in nesting females when compared to neonates at emergence (27X) and 3–4 weeks (81X). The phylum Patescibacteria is considered a superphylum composed of a diverse set of bacteria (Rinke et al., 2013; Haro-Moreno et al., 2023). Bacteria within this phylum are often found in aquatic environments (Leon-Zayas et al., 2017; Rahlff et al., 2020; Zhao et al., 2022; Haro-Moreno et al., 2023). Thus, it is likely that the abundance of this bacterial phylum on the skin increases with prolonged exposure to an aquatic environment (i.e., seawater) and therefore is found in higher abundance on the skin of nesting females. At the family level, there is a shift in the two most abundant families from Xanthomonadaceae and Moraxellaceae at emergence to Flavobacteriacea and Rodobacteraceae

at 3–4 weeks of age. This shift in the most abundant bacterial families most likely reflects a normal life-stage class related change in skin microbiota and is also likely related to the environmental shift the neonate makes from the nest environment to the ocean. Nest sand microbiota has the potential to influence the microbiota present on eggs and therefore likely the hatchling (McMaken et al., 2023). Additionally, life stage and local environment are known to influence microbial communities (Ying et al., 2025; Price et al., 2017; Ross et al., 2018; Scheelings et al., 2020b). These theories are further supported as the most prominent bacterial families on nesting females are also Flavobacteriacea and Rodobacteraceae.

Observations in alpha diversity reveal significant differences in the microbial diversity on individuals at each life-stage class. All measures of alpha diversity (i.e., Observed, Chao1, Shannon, and Inverse Simpson) reveal a trend of increasing alpha diversity over time. Additionally, the significant differences observed in beta diversity suggest that the communities present at each life-stage class are different. This suggests that the microbial communities present on the skin changes throughout the life-stage classes measured here. Limited studies in reptiles make comparisons difficult; however, studies in humans have reported changes in skin microbiota related to age (Leyden et al., 1975; Capone et al., 2011; Ying et al., 2015; Howard et al., 2022).

It is important to note that the leatherback neonates at 3–4 weeks of age included in this study were maintained in a laboratory environment. The ocean water the neonates are housed in is filtered and treated with UV light to kill any microbes present. Additionally, for biosecurity reasons all neonates are handled minimally, and only using clean examination gloves. Consequently, the maintenance of these animals in this ‘clean’ environment likely has some impact on the skin microbiota. It has also been shown that captivity can impact the composition of microbiota (Becker et al., 2014; Cheng et al., 2015; Bataille et al., 2016). Consequently, the microbiota data presented here for leatherback neonates at 3–4 weeks of age may not directly reflect their wild counterparts. However, due to limitations with accessing wild populations of neonatal leatherbacks, this is the closest approximation to healthy wild leatherback neonates currently available. In the future, if access allows, additional studies should be conducted on wild leatherback neonates. Though access to wild leatherback neonates is limited, a potential alternative next step to consider could be rearing leatherbacks in untreated and constantly circulating ocean water.

Comparisons between carapace and flipper on nesting females

The relative abundance of both bacterial phyla and families were relatively similar between the carapace and the flipper of nesting females. However, the phyla Cyanobacteria and Verrucomicrobiota were both more predominant on the flipper while Patescibacteria was found in higher abundance on the carapace. The bacterial family Rubritaleaceae was notably more abundant on the flipper. Alpha diversity was consistently higher on the flipper when compared to the carapace and differences in beta diversity between locations show that the bacterial communities at each location were significantly different. Minor differences in relative abundance of phyla and families were expected, as were the significant differences in both alpha and beta diversity.

Previous studies in loggerheads and freshwater turtles have demonstrated significant differences in microbial communities exist on different parts of the body such as the head, skin,

and shell (McKnight et al., 2020; Parks et al., 2020; Kanjer et al., 2022). Kanger et al., (2022) reported higher diversity and richness on the carapace of loggerheads when compared to samples taken from the skin (i.e., head, neck, and flippers). This is likely due to microhabitats on the skin that influence the microbiota at different locations on the body (Bataille et al., 2016). In contrast to the loggerheads, I found significantly higher alpha diversity on the flippers of leatherbacks when compared to the carapace. This difference is likely due to the unique composition of the leatherbacks integumentary system. The adult leatherback carapace is a waxy, pliable surface composed of a resilient and flexible alpha-keratin underlying a thin layer of beta-keratin (Boylan et al., 2017). In contrast to this the flippers are covered in smooth and fine scalation (Boylan et al., 2017). The texture and compositional differences between these two regions likely create two unique microhabitats that may be more or less favorable to particular bacteria. The fine scalation on the front flipper likely allows the proliferation of a more diverse array of bacteria resulting in the higher alpha diversity observed.

Health assessments of nesting sea turtles are the most common due to the ease of accessing them during oviposition (Deem et al., 2006; Perrault et al., 2012, 2014; Stacy et al., 2019). However, it is important to note that the physiologic state of the nesting female is highly unique and may influence the skin microbiota (Ikonomopoulou et al., 2014; Park et al., 2022; Rubin et al., 2023). Leatherback sea turtles are capital breeders that forage little to none during the nesting season (Plot et al., 2013; Perrault et al., 2014). In addition to fasting a nesting female also undergoes changes in hormone levels associated with reproduction (Ikonomopoulou et al., 2014; Park et al., 2022; Rubin et al., 2023), which has been shown to impact skin microbiota in humans (Park et al., 2022; Ma et al., 2023). Consequently, the microbiota data presented here for nesting leatherbacks may not reflect the normal microbiota of a foraging leatherback (Kuschke, 2022). As such, additional studies should be aimed at characterizing the normal microbiota of foraging leatherbacks, both male and female.

The differences between sample locations seen in nesting females may also be true for neonatal leatherbacks. In this study, the whole body of neonatal leatherbacks was swabbed due to their small size and the generally conserved fine scalation across their body (Boylan et al., 2017). However, given the observations in nesting females, differences between sample locations (i.e., carapace and flipper) should also be explored over time in neonates. As leatherback neonates grow and shed their scales, it is likely that differences in microbial communities will arise across the body and resemble that of the nesting female. The shift of these communities over time could then be used to make inferences to the role of the bacteria at different locations and during different life stages.

Conclusion

This study presents novel information on leatherback skin microbiota and reveals that a unique skin microbiota exists at different life-stage classes. Additionally, I found that different locations on the body host unique microbial communities. Moving forward, additional research should be aimed at identifying the core microbiota present on the skin or foraging leatherbacks and in wild neonates, if logistically feasible. However, these baseline data serve as an essential foundation to begin to understand the role that the skin microbiota plays in leatherback sea turtle health, immune function, disease, and resilience to environmental stressors. Understanding these

essential host-microbial relationships is the next step in managing this highly endangered species.

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Appendix

Table 3.1 Summary of sequencing results

**This number includes swabs from both the flipper and carapace.*

	Turtles sampled	**Number of samples submitted for PCR and NGS**	**Samples used for analysis**	**Total reads**	**Average reads**
Neonates at emergence	178	172	151	7,503,179	49,690
3-4 weeks	140	135	126	7,337,226	58,232
Nesting females	37	74*	63	4,767,105	74,486

Table 3.2 Relative abundance of top 10 most abundant bacterial phyla across life-stage classes

Phylum	**Neonates at emergence**	**Neonates at 3–4 weeks**	**Nesting females**
Proteobacteria	63.0	67.5	60.6
Bacteroidota	26.2	23.8	25.3
Actinobacteriota	6.7	4.5	1.6
Patescibacteria	0.3	0.1	8.1
Bdellovibrionota	2.5	0.3	0.8
Firmicutes	0.8	2.2	0.2
Cyanobacteria	0.01	0.03	1.4
Planctomycetota	0.06	1.1	0.16
Verrucomicrobiota	0.39	0.08	0.97
Myxococcota	0.06	0.18	0.02

Table 3.3 Relative abundance of top 10 most abundant bacterial families across life-stage classes

Family	**Neonates at emergence**	**Neonates at 3–4 weeks**	**Nesting females**
Flavobacteriacea	5.7	21.1	50.1
Rhodobacteraceae	3.0	13.3	17.9
Xanthomonadaceae	14.2	7.0	0.02
Moraxellaceae	13.3	0.3	2.1
Alcaligenaceae	6.3	0.95	0.002
Pseudomonaadaceae	9.1	4.6	0.04
Weeksellaceae	10.8	1.3	1.6
Enterobacteriaceae	2.6	10.8	.0008
Rhizobiaceae	5.5	2.4	.04
Nocardiaceae	3.8	3.6	.004

Table 3.4 Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons across alpha diversity measured by Observed OTUs revealed significant differences among all groups (df = 2).

	Emergence	3–4 weeks
3–4 weeks	0.003	--
Nesting females	<0.001	<0.001

Table 3.5 Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons across alpha diversity measured by Chao1 indices revealed significant differences among all groups (df = 2).

	Emergence	3–4 weeks
3–4 weeks	0.003	--
Nesting females	<0.001	<0.001

Table 3.6 Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons across alpha diversity measured by Shannon indices revealed significant differences among all groups (df = 2).

	Emergence	3–4 weeks
3–4 weeks	<0.001	--
Nesting females	<0.001	<0.001

Table 3.7 Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons across alpha diversity measured by Inverse Simpson indices revealed significant differences among all groups (df = 2).

	Emergence	3–4 weeks
3–4 weeks	<0.001	--
Nesting females	<0.001	<0.001

Table 3.8 P-values for the pairwise Adonis tests used to compare beta diversity between the life-stage classes.

	Emergence	3–4 weeks
3–4 weeks	<0.001	--
Nesting females	<0.001	<0.001

Table 3.9 Relative abundance of top 10 bacterial phyla of different sample locations on nesting females.

Phylum	**Carapace**	**Flipper**
Proteobacteria	63.9	57.5
Bacteroidota	22.2	28.2
Patescibacteria	10.8	5.6
Actinobacteriota	1.3	1.8
Cyanobacteria	0.48	2.2
Verrucomicrobiota	0.09	1.8
Bdellovibrionota	0.49	1.2
Deinococcota	0.02	1.0
Campilobacterota	0.55	0.04
Firmicutes	0.09	0.25

Table 3.10 Relative abundance of the top 10 bacterial families of different sample locations on nesting females

Family	**Carapace**	**Flipper**
Flavobacteriacea	58.9	41.8
Rodobacteraceae	17.5	18.3
Saprospiraceae	12.6	13.3
Moraxellaceae	1.4	2.9
Rubritaleaceae	0.04	3.9
Hyphomonadaceae	1.1	1.9
Bdellovibrionaceae	0.56	2.4
Weeksellaceae	0.22	2.8
Crocinitomicaceae	0.80	1.5
Trueperaceae	0.03	2.3

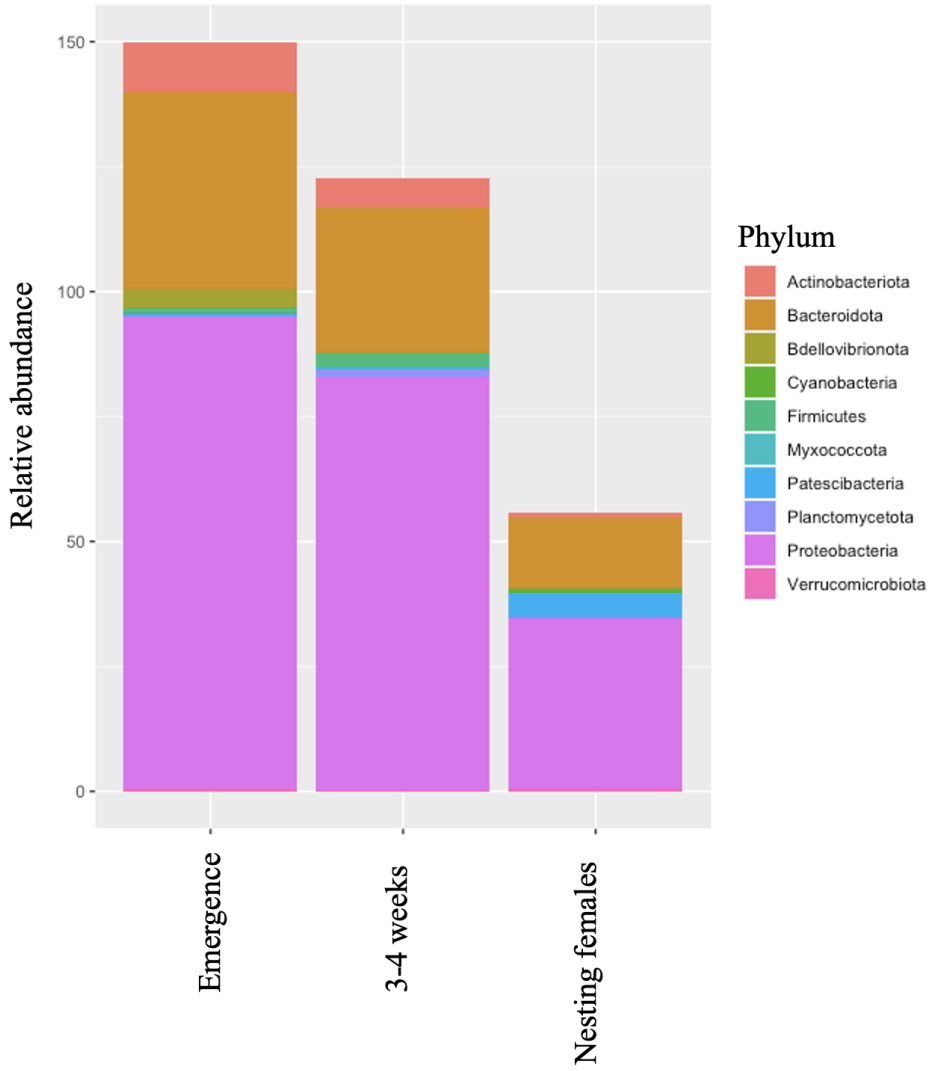


Figure 3.1 Relative abundance of top 10 bacterial phyla in different life-stage classes of leatherback sea turtles. Note that at the phylum level there are few differences between the life-stage classes. However, Patescibacteria is more prominent on the nesting females when compared to neonates at emergence or 3–4 weeks of age.

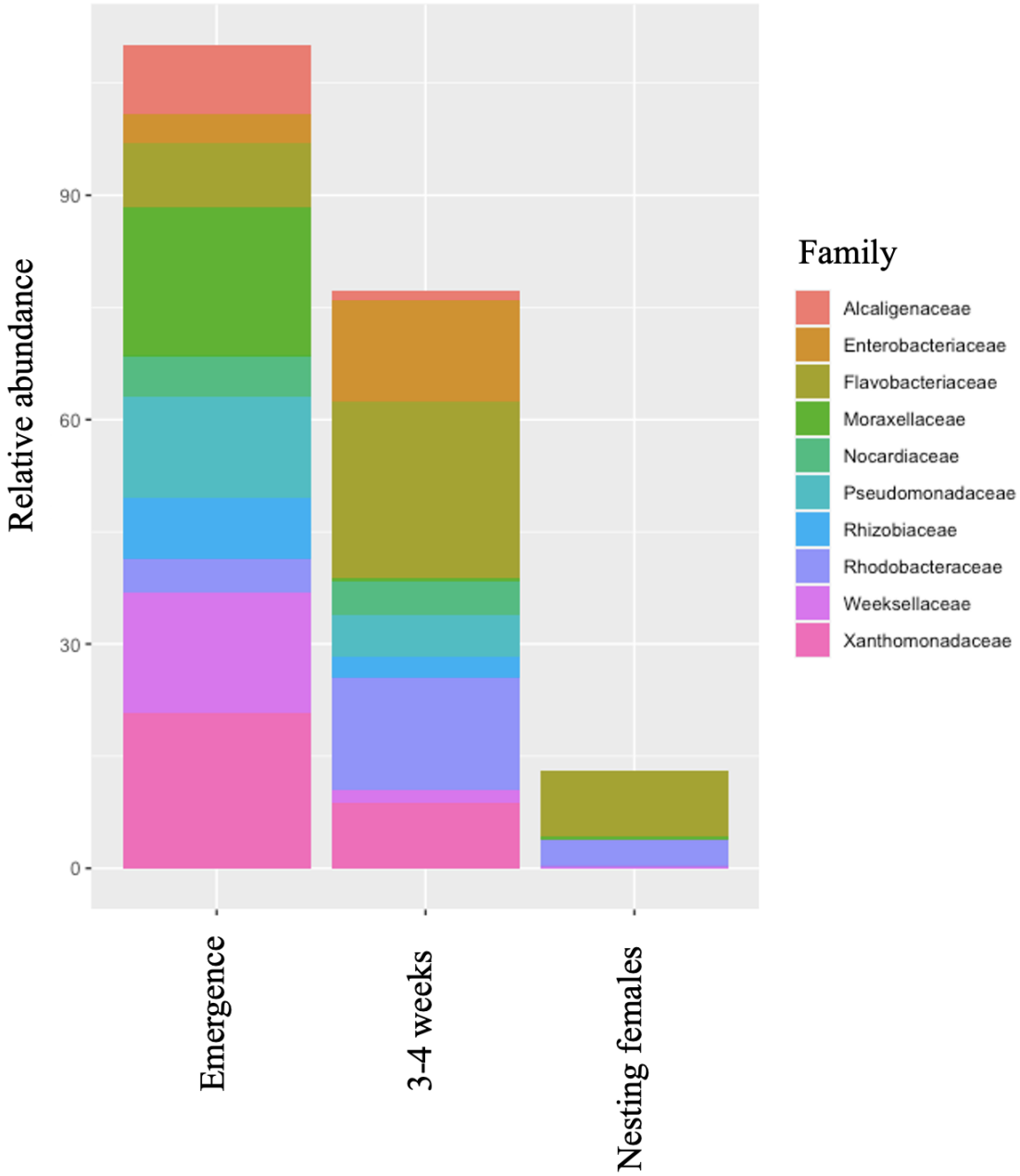


Figure 3.2 Relative abundance of top 10 bacterial families in different life-stage classes of leatherback sea. A broad range on bacterial families was found on neonates at emergence with the most abundant families being Xanthomonadaceae and Moraxellaceae. In neonates at 3–4 weeks, the most abundant families shifted to Flavobacteriaceae and Rodobacteraceae. Flavobacteriaceae and Rodobacteraceae were also the most abundant bacterial families found on the nesting females.

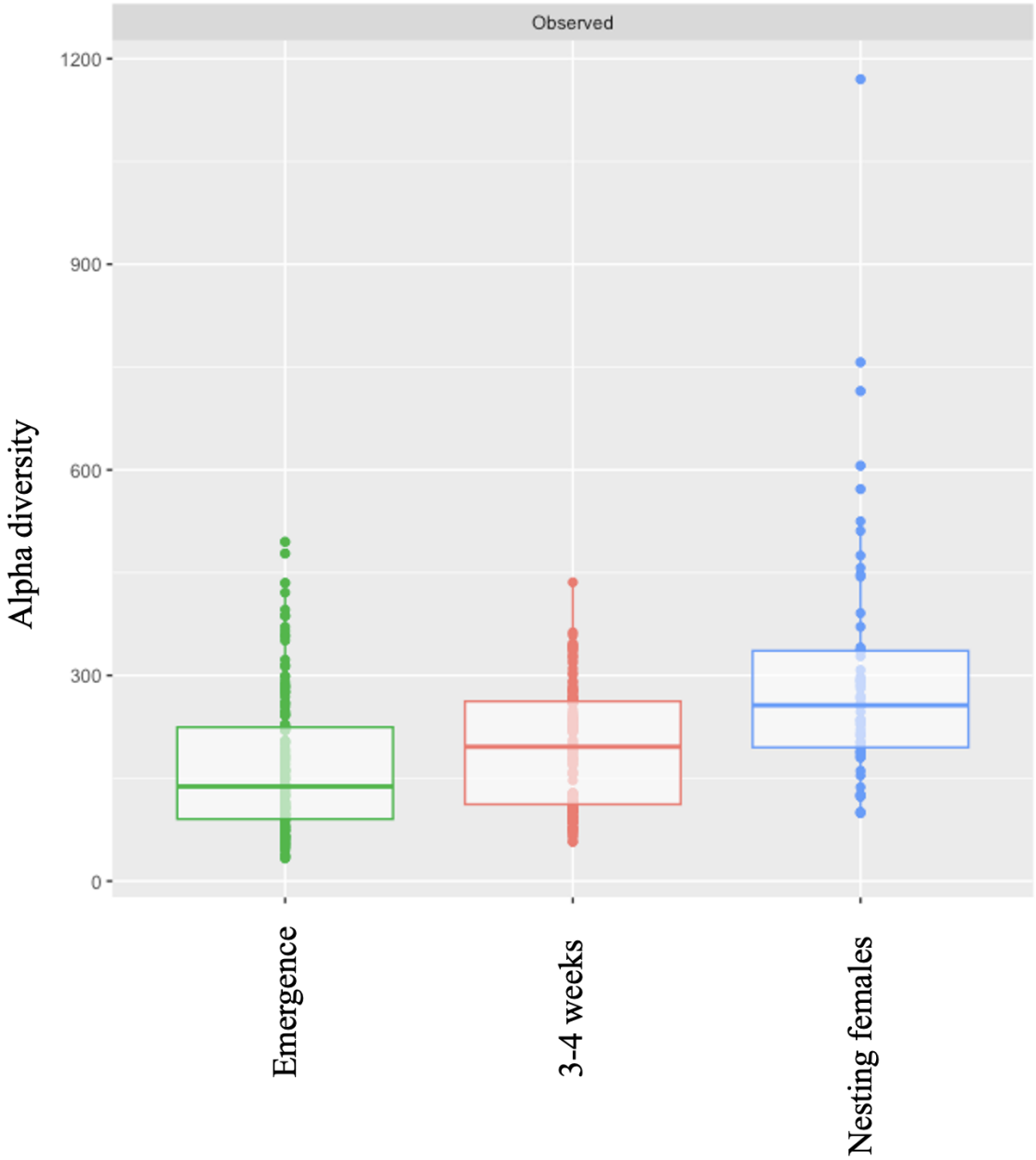


Figure 3.3 Alpha diversity across life-stage classes of leatherback sea turtles measured by Observed OTUs. Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons revealed significant differences among all groups (df = 2). *See Table 3.4 for p-values; Boxes are the first and third quartile; the line in the center of the box is the mean; the whiskers are the minimum and maximum values*

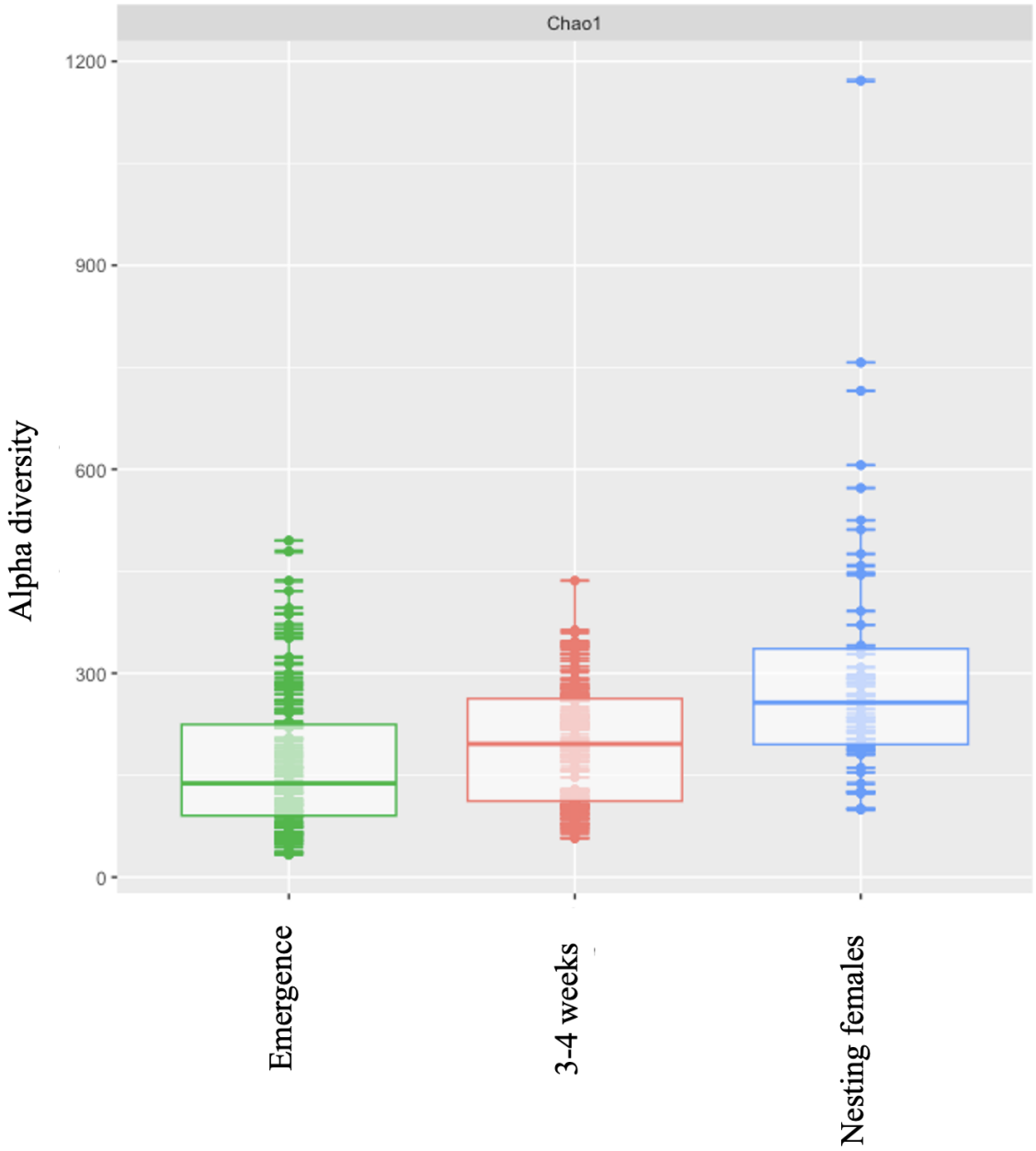


Figure 3.4 Alpha diversity across life-stage classes of leatherback sea turtles measured by Chao1 indices. Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons revealed significant differences among all groups (df = 2). *See Table 3.5 for p-values; Box-plot format as in Figure 3.3*

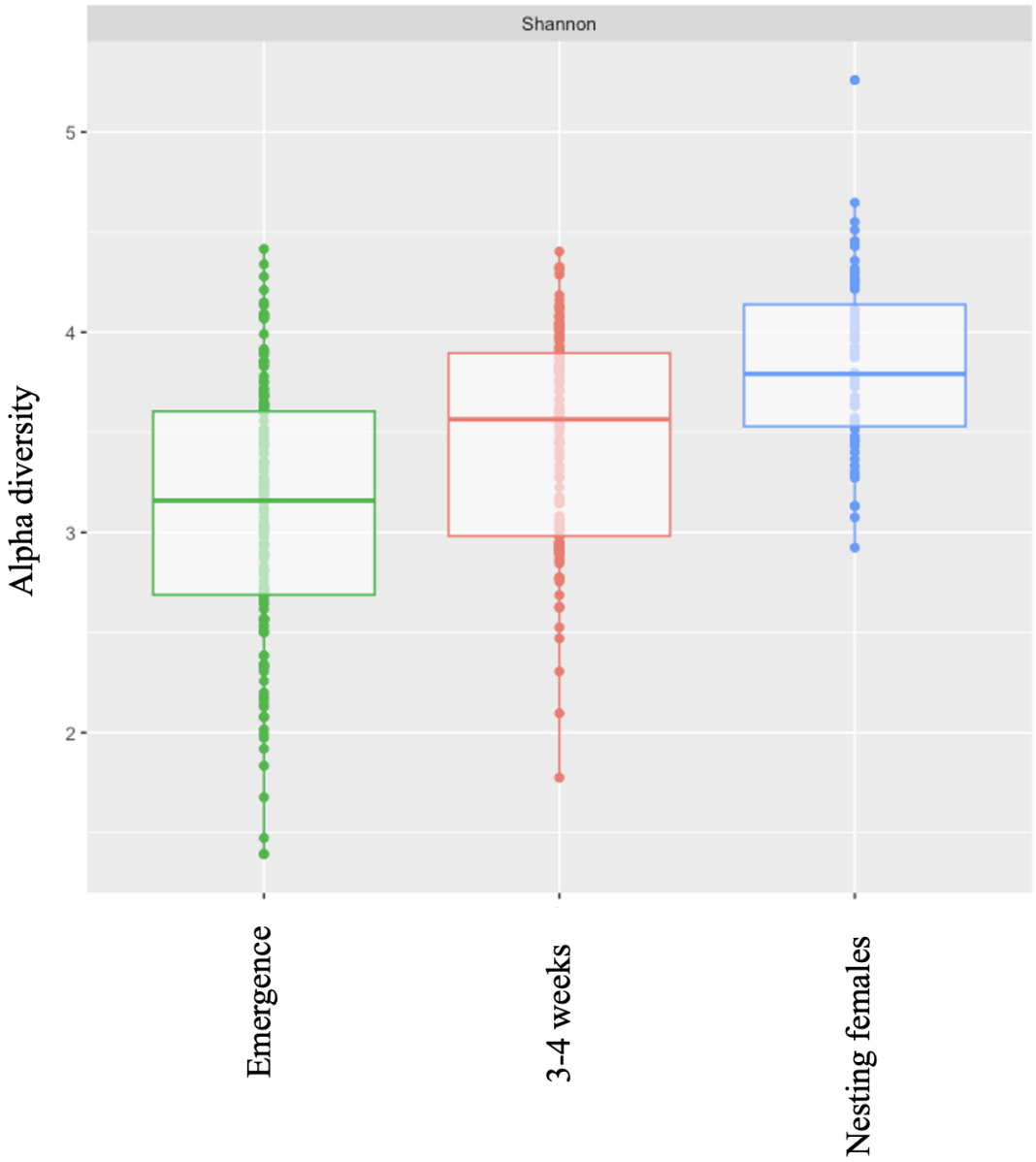


Figure 3.5 Alpha diversity across life-stage classes of leatherback sea turtles measured by Shannon indices. Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons revealed significant differences among all groups (df = 2). *See Table 3.6 for p-values; Box-plot format as in Figure 3.3*

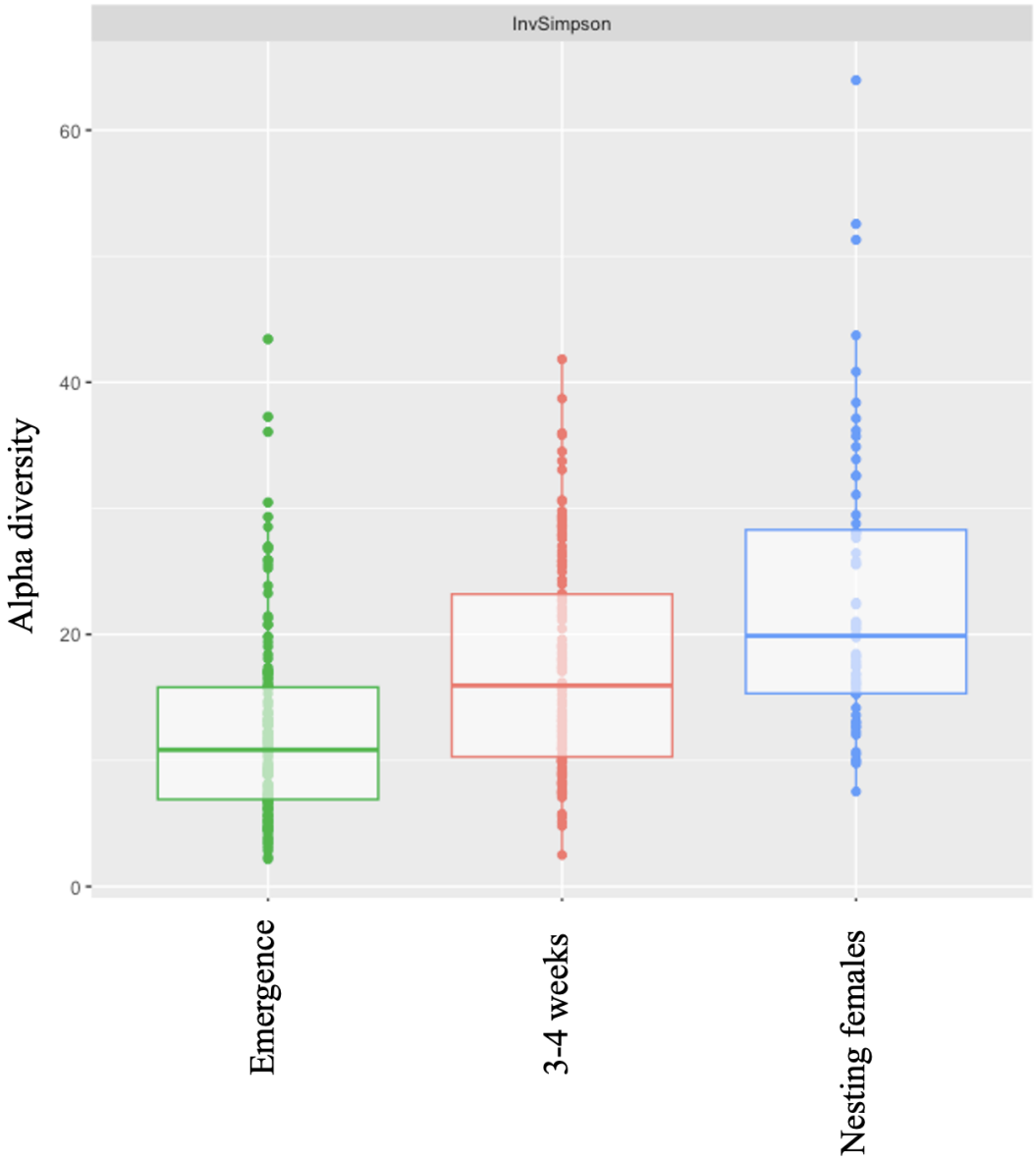


Figure 3.6 Alpha diversity across life-stage classes of leatherback sea turtles measured by Inverse Simpson indices. Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons revealed significant differences among all groups (df = 2). *See Table 3.7 for p-values; Box-plot format as in Figure 3.3*

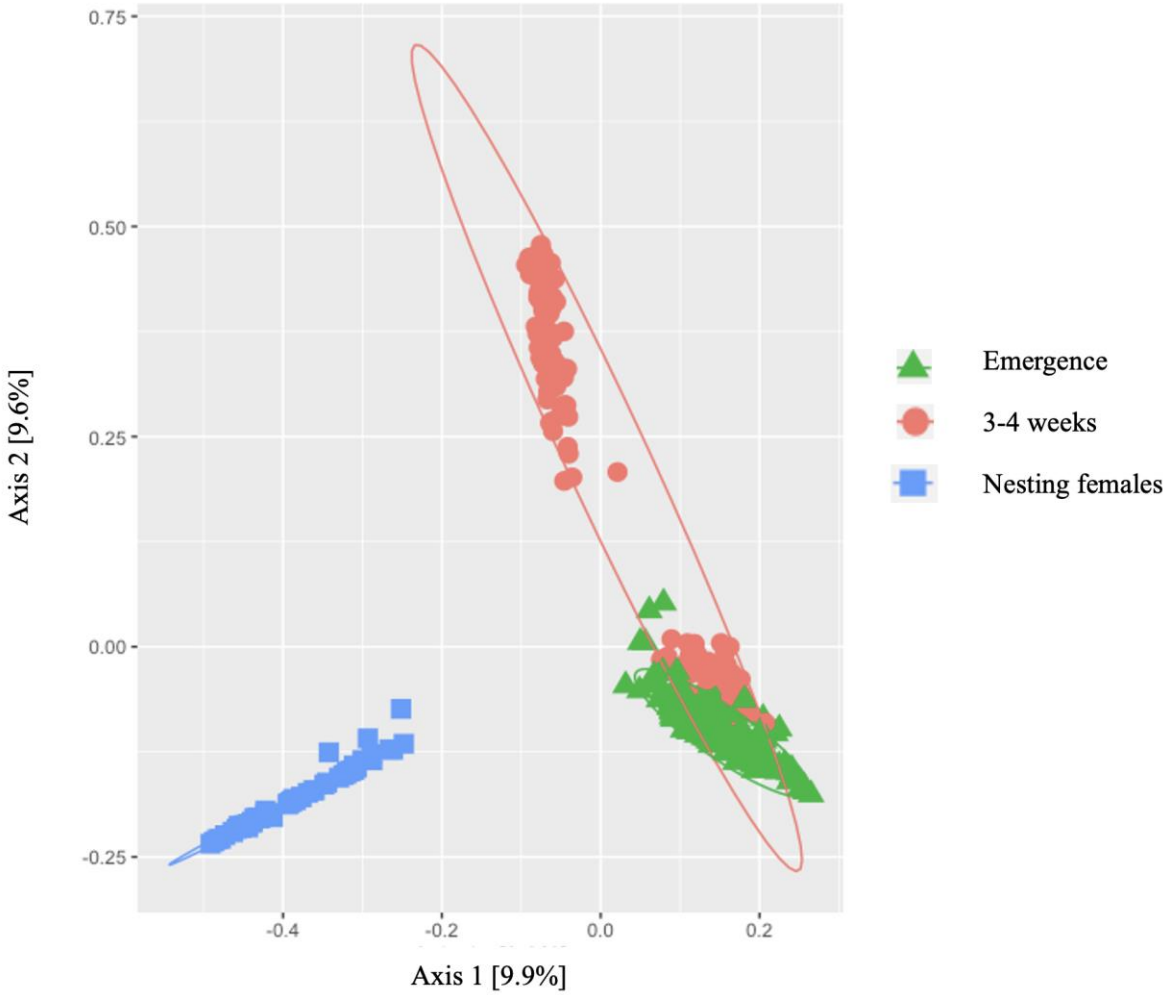


Figure 3.7 Beta diversity expressed using a principal coordinate analysis (PCoA) of Bray-Curtis distance showing differences in microbial diversity across all life-stage classes of leatherback sea turtles. Significant differences exist in microbial communities between all life-stage classes (see Table 3.8 for p-values). There is some overlap between neonates at emergence and neonates at 3-4 weeks. This may be due to a slower shift in the microbial community in some individuals. However, significant differences were still seen between all life-stage classes regardless of the overlap in neonates.

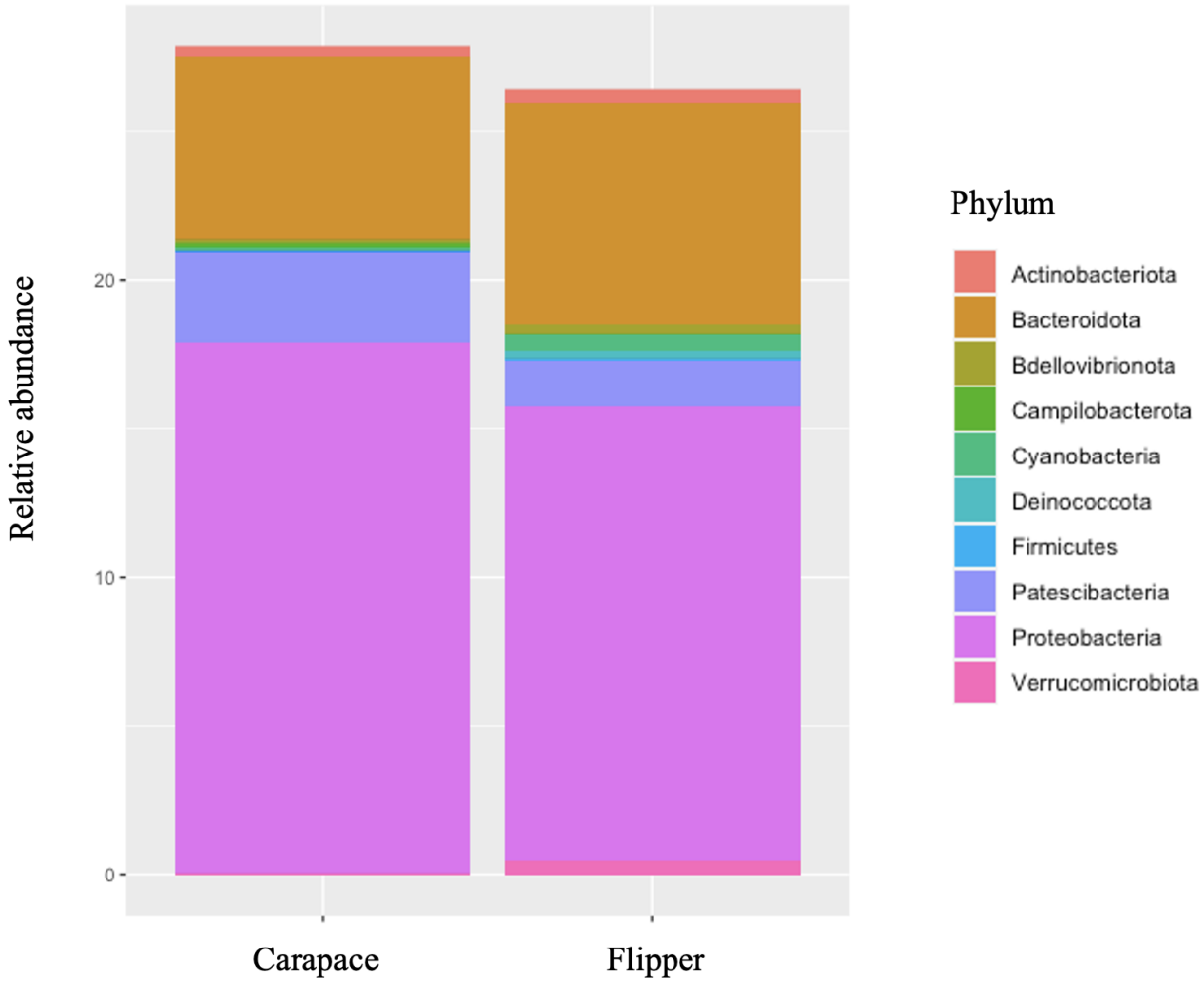


Figure 3.8 Relative abundance of bacterial phyla on the carapace and flipper of nesting leatherback sea turtles. Note that Patescibacteria is more abundant on the carapace than on the flipper.

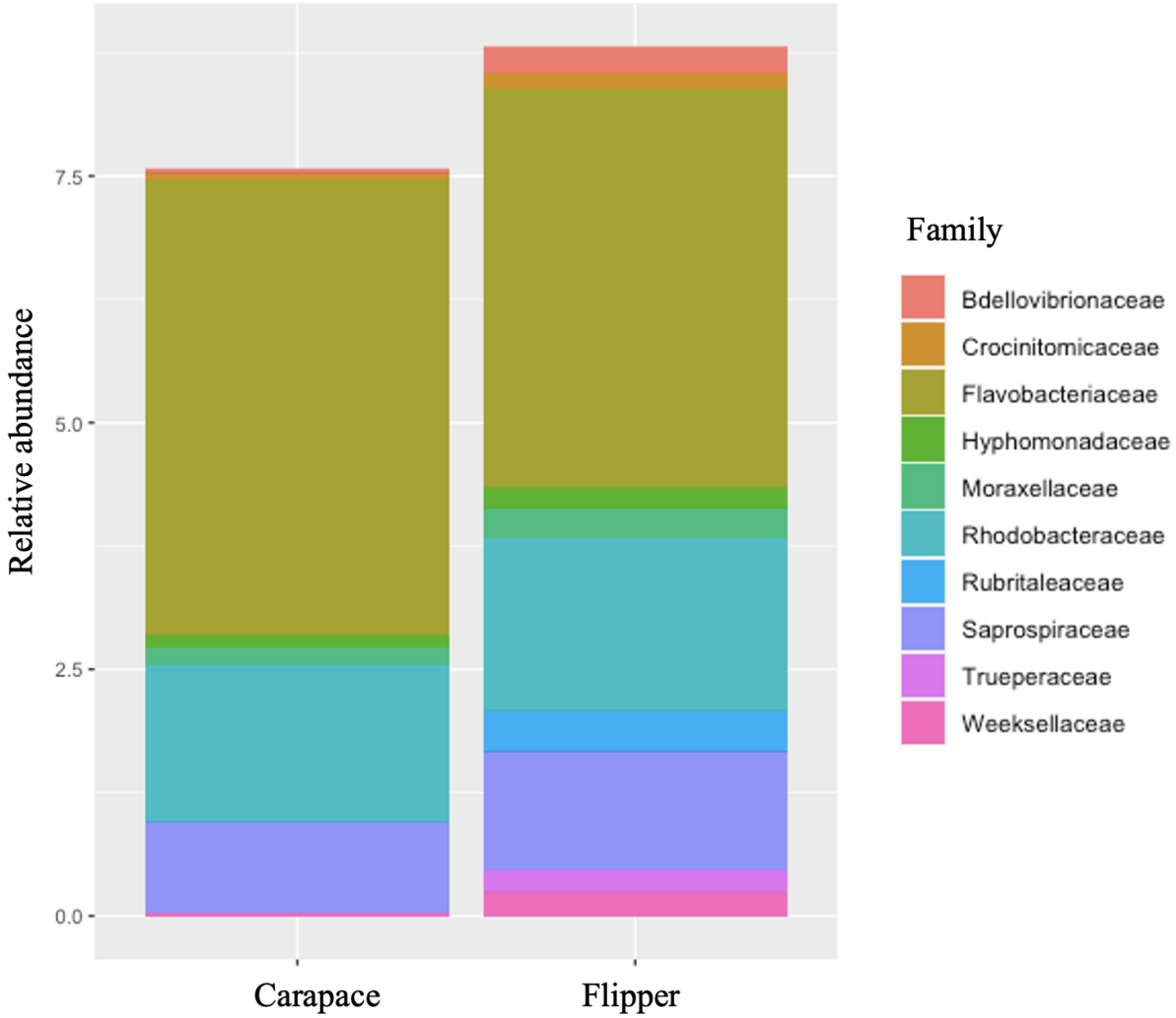


Figure 3.9 Relative abundance of bacterial families on the carapace and flipper of nesting leatherback sea turtles. Note that Rubritaleaceae is present in higher abundance on the flipper than on the carapace.

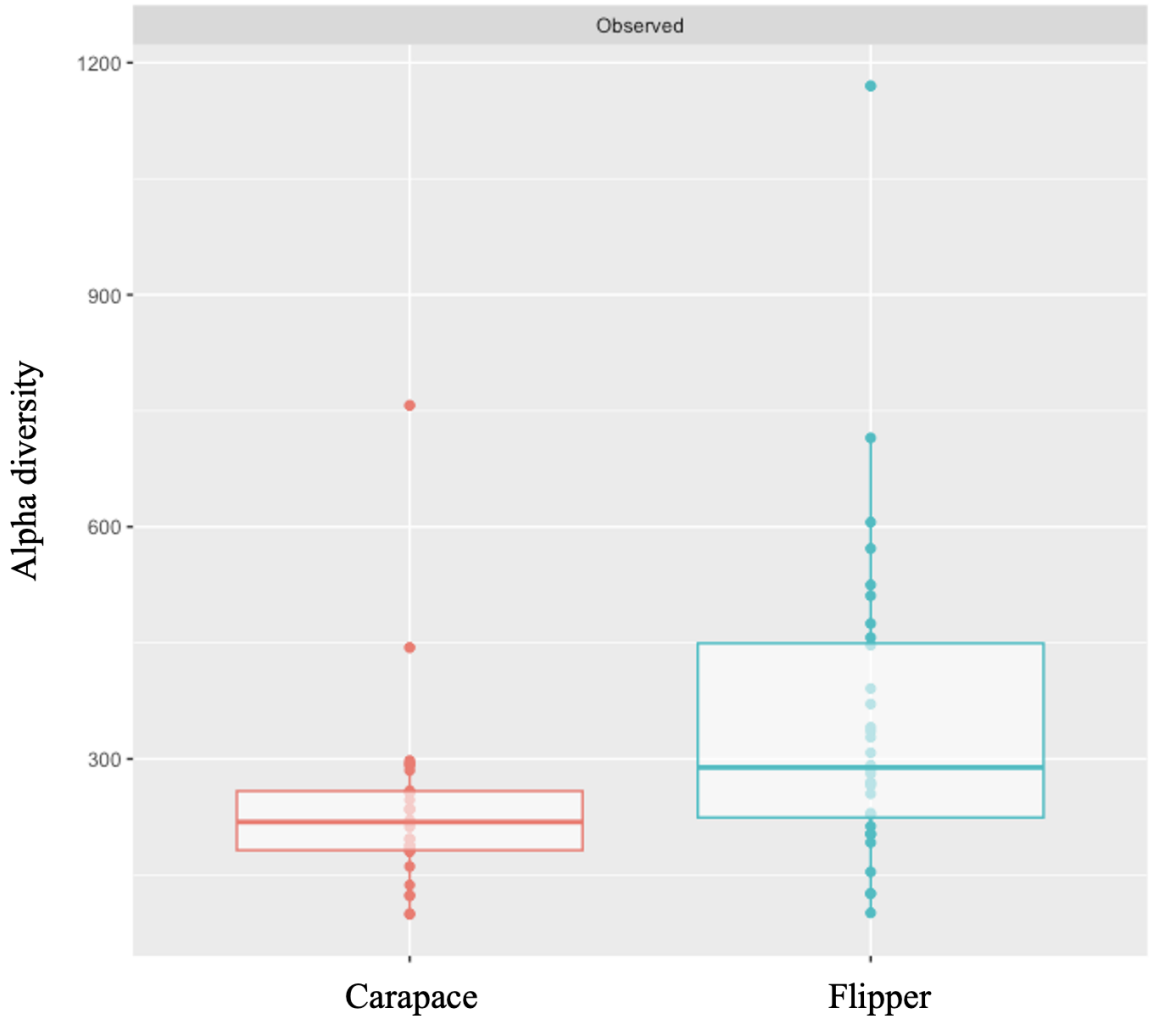


Figure 3.10 Alpha diversity of the carapace and flipper of nesting leatherbacks measured by Observed OTUs. Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons revealed significant differences between groups (df = 1, p = 0.004). *Box-plot format as in Figure 3.3*

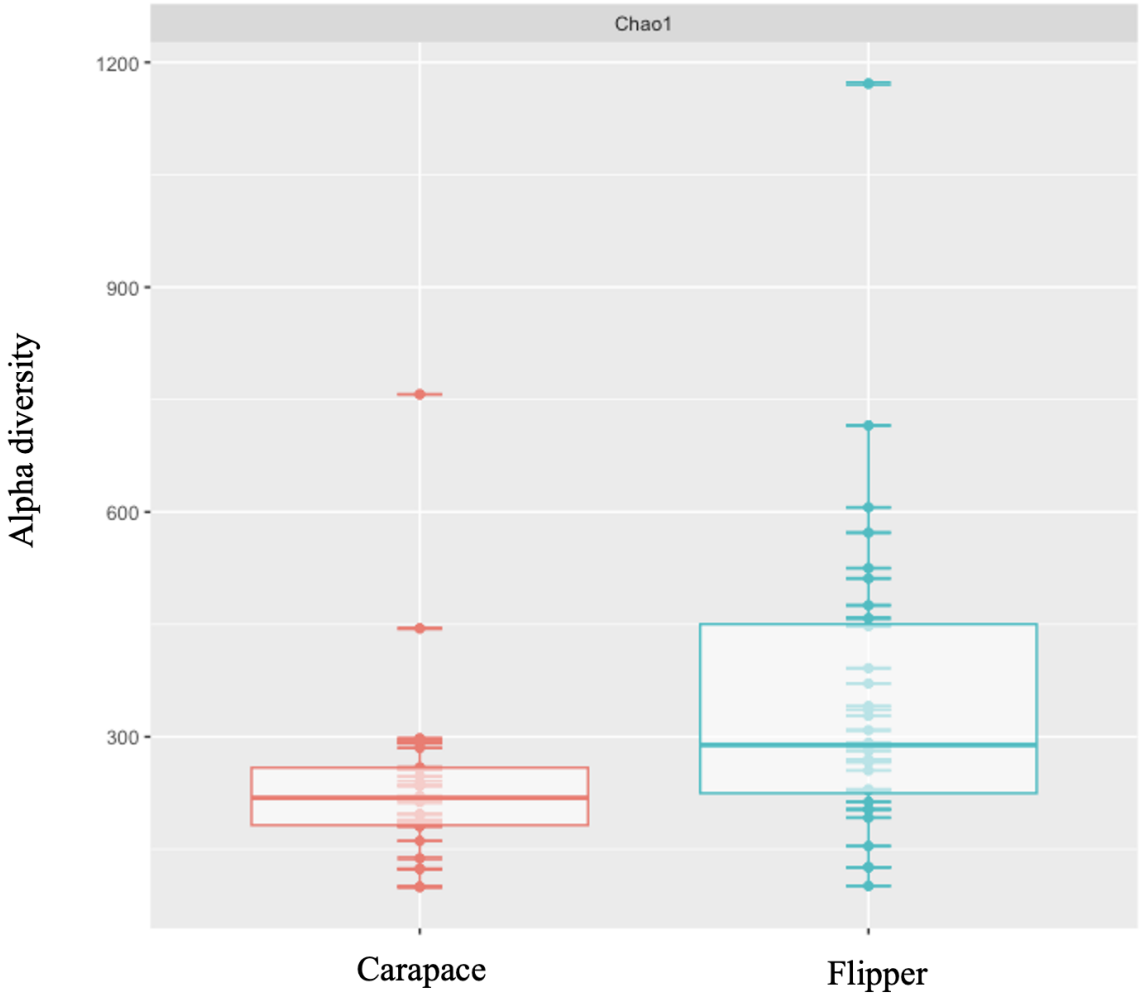


Figure 3.11 Alpha diversity of the carapace and flipper of nesting leatherback sea turtles measured by Chao1 indices. Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons revealed significant differences between groups (df = 1, p = 0.004). *Box-plot format as in Figure 3.3*

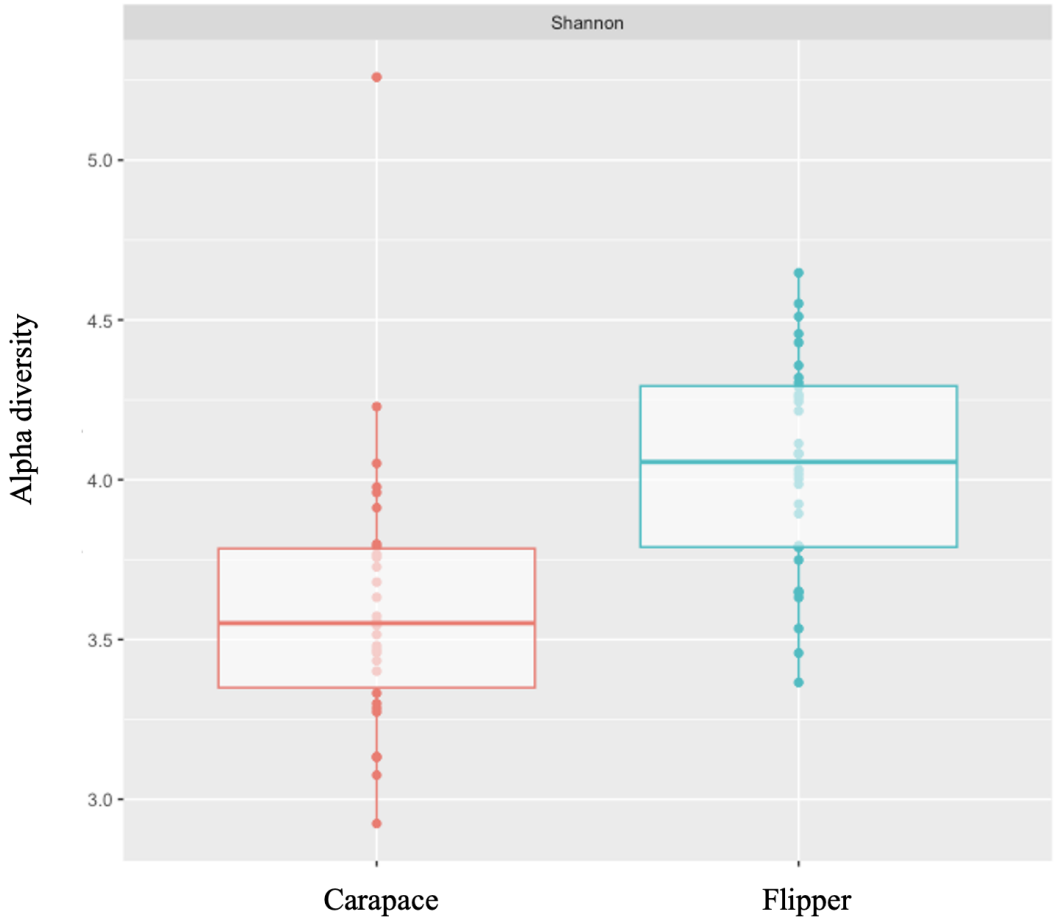


Figure 3.12 Alpha diversity of the carapace and flipper of nesting leatherbacks measured by Shannon indices. Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons revealed significant differences between groups ($df = 1$, $p < 0.001$). *Box-plot format as in Figure 3.3*

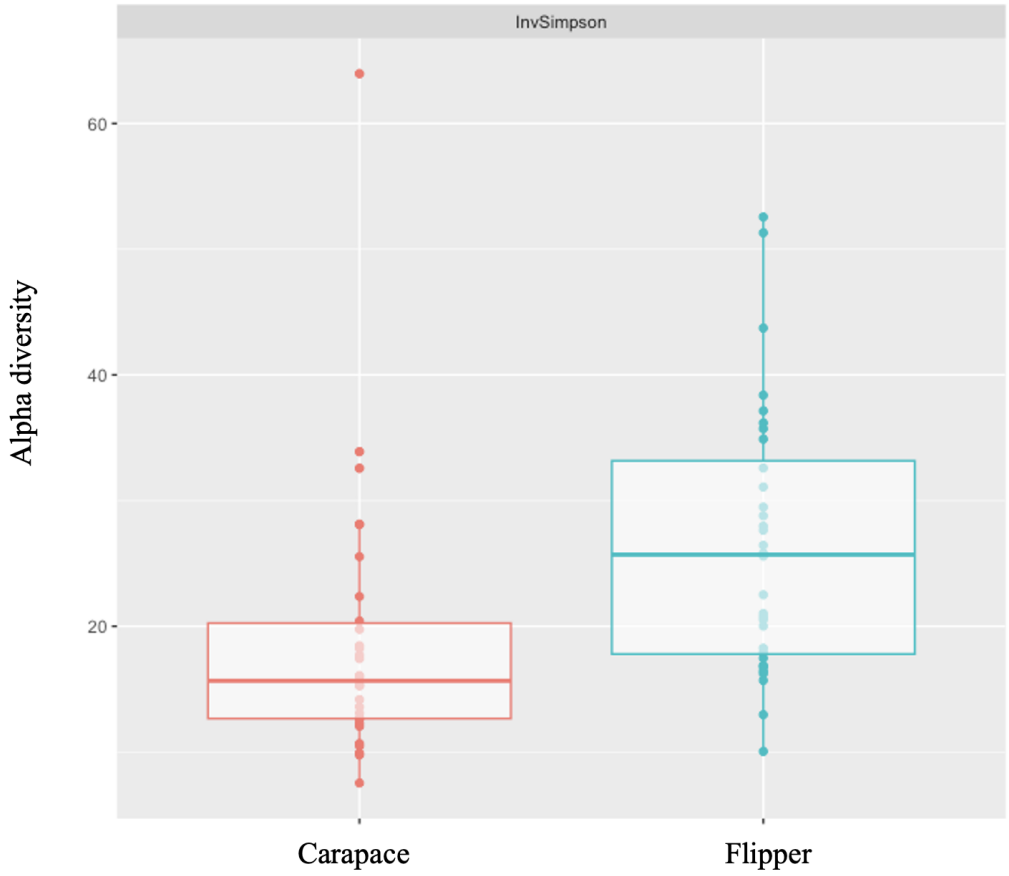


Figure 3.13 Alpha diversity of the carapace and flipper of nesting leatherbacks measured by Inverse Simpson indices. Pairwise Wilcoxon rank sum tests with Holm p-values adjusted for multiple comparisons revealed significant differences between groups ($df = 1$, $p < 0.001$). *Box-plot format as in Figure 3.3*

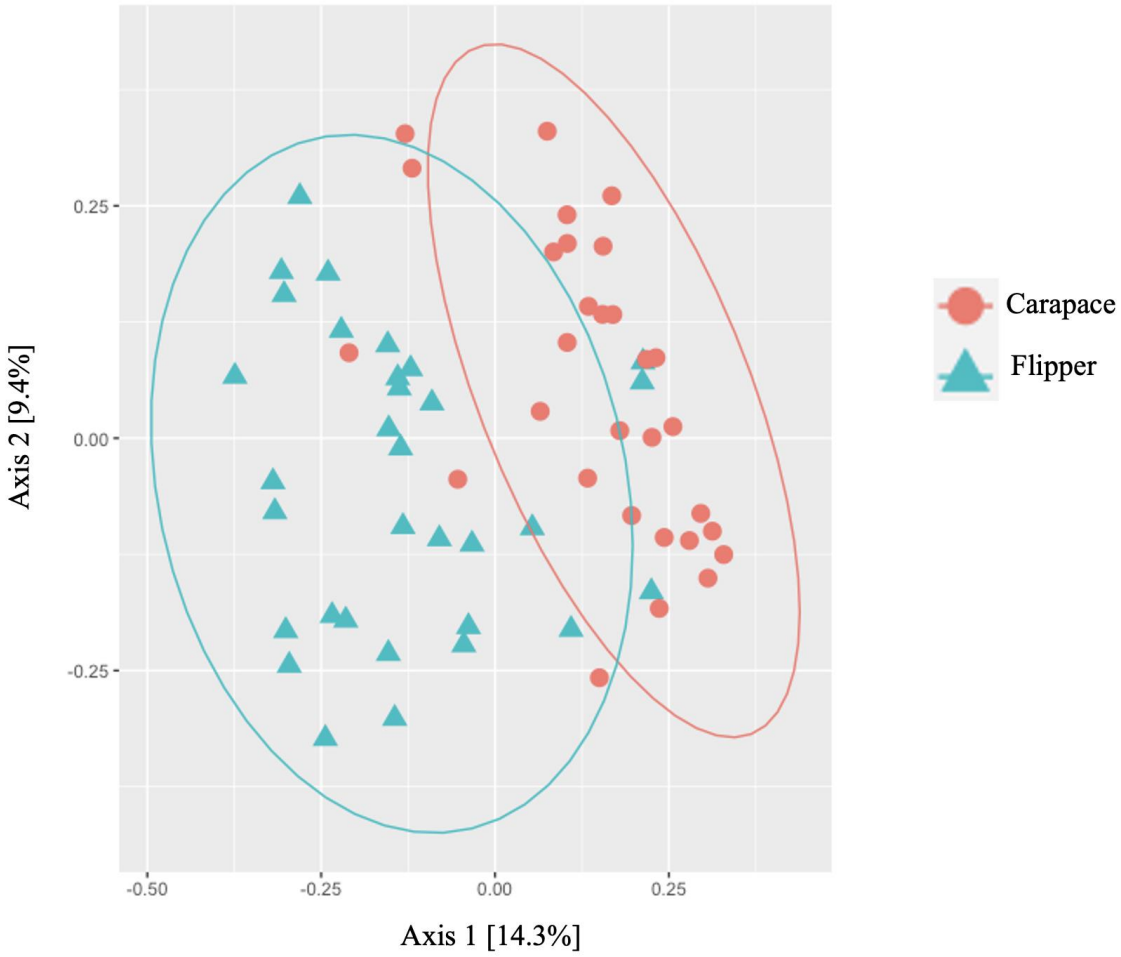


Figure 3.14 Beta diversity expressed using a principal coordinate analysis (PCoA) of Bray-Curtis distance showing differences in microbial diversity between the carapace and flipper on nesting leatherback sea turtles. Significant differences in microbial communities existed between the two sample locations ($R^2 = 0.09$, $p = .001$).

CHAPTER IV IMPACTS OF RISING INCUBATION TEMPERAURES ON NEONATAL LEATHERBACK SEA TURLTE SKIN MICROBIOTA

Abstract

In the last decade, climate change has been negatively impacting the health of humans, animals, and plants around the world. Marine animals, such as sea turtles, have been exceedingly impacted by climate change as it impacts multiple life-stage classes. Sea turtles are facing challenges related to climate change both on the beach where they lay their eggs and in the ocean. Recent studies have established that increasing incubation temperatures negatively impact neonatal sea turtles at emergence, however none have studied the impact on the microbiota. Due to constant contact with the outside world the skin microbiota is significantly influenced by external factors like rising incubation temperatures. Here I present novel data exploring the impact of incubation temperature on the skin microbiota of leatherback sea turtle (*Dermochelys coriacea*) neonates at emergence and at 3–4 weeks of age. I found significant differences in the skin microbiota of leatherback neonates that incubated in hot nests versus those that incubated in cooler nests. Significant differences between these groups were still present at 3-4 weeks of age. This suggests that the impacts of increased incubation temperature may occur longer than expected and could ultimately negatively impact neonatal leatherback health after emergence from the nest.

Introduction

One of the major environmental factors that has been impacting humans, animals, and plants worldwide is climate change (Root et al., 2003; Anderson et al., 2004; Simmonds and Isaac 2007; Rosenzweig et al., 2008; Chen et al., 2011; Pörtner and Gutt 2016; Fleming et al., 2020; Perrault et al., 2022). More specifically increasing environmental temperatures are negatively impacting marine animals (Simmonds and Isaac 2007; Brierley and Kingsford 2009; Marcogliese 2008; Pörtner and Gutt 2016; Fleming et al., 2020; Perrault et al., 2022). Increasing temperatures are causing shifts in species distributions, changing beach structures, altering interspecies competition, and negatively impacting marine animal health (Brierley and Kingsford 2009; Marcogliese 2008; Pörtner and Gutt 2016; Fleming et al., 2020; Perrault et al., 2022, Seaman and Milton 2023). These negative impacts are even more extreme in marine animals like sea turtles that utilize beaches, as they must combat both increasing ocean and beach temperatures (NMFS and USFWS, 2020). Consequently, many sea turtle species currently face an increased risk of extinction partly due to the negative impacts of climate change (NMFS and USFWS, 2020).

The northwestern Atlantic population of leatherback sea turtles (*Dermochelys coriacea*) is considered to be at high risk of extinction due to decreasing nest productivity (NMFS and USFWS, 2020). The cause of leatherback conservation status in this region is multifactorial (NMFS and USFWS, 2020). However, due to its impact on all life-stage classes of leatherbacks including their eggs, rising global temperatures are significantly impacting the northwestern Atlantic leatherback population (NMFS and USFWS, 2020). Beaches play an essential role in the reproductive success and ultimately success of leatherback populations (Bolten et al., 2011). Sea turtle eggs are especially susceptible to shifts in temperature as they incubate in the sand without parental care (Bolten et al., 2011). Consequently, reproductive success is highly dependent upon environmental conditions (Bolten et al., 2011). Currently, the full impact of increased incubation temperatures on leatherback eggs and neonates at emergence is not fully understood (Mickelson and Downie 2010; Rivas et al., 2019; Perrault et al., 2022; Seaman and

Milton 2023). However, recent studies have observed alterations in neonatal morphology, performance, and blood analytes that indicate increasing temperatures are negatively impacting neonatal health and thus survival after emerging from the nest (Fleming et al., 2020; Page-Karjian et al., 2022; Perrault et al., 2022; Seaman and Milton 2023).

Recently, research exploring the microbiota on and within humans and other animals has increased (Isler et al., 2013; Sommer and Backhed 2013; Selosse et al., 2014; Colston and Jackson 2016; Walke and Belden 2016; Simon et al., 2019), showing that the microbiota is essential to supporting overall health (Sommer and Backhed 2013; Selosse et al., 2014; Colston and Jackson 2016; Walke and Belden 2016; Simon et al., 2019). Microorganisms that make up the microbiota are known to play key roles in immune function, host resistance, and aid in the hosts response to internal and external stressors (Harris et al., 2009; Selosse et al., 2014; Walke et al., 2015a; Colston and Jackson 2016; Walke and Belden 2016; Jiménez and Sommer 2016; Kho and Lal 2018; Simon et al., 2019). More specifically, the skin microbiota serve as the first line of defense against the outside world and harmful elements encountered within it (Isler et al., 2023). The skin microbiota directly competes and inhibits the growth of pathogens on the skin and aids in both adaptive and innate immunity (Sanford and Gallo 2013). Due to its constant contact and interaction with the environment, the skin microbiota can be highly influenced by external factors such as increasing temperatures (Ghosh et al., 2022; Yang et al., 2022a; Isler et al., 2023). Recent studies have found that increased environmental temperatures can disrupt the microbiota present on the skin (Gosh et al., 2022; Yang et al., 2022a; Isler et al., 2023). These disruptions are theorized to negatively impact host health (Gosh et al., 2022; Yang et al., 2022a; Isler et al., 2023). Currently, there are few studies exploring the skin microbiota in sea turtles, and no studies investigate the impact of increased incubation temperatures on the skin microbiota of sea turtle neonates (Kuschke, 2022). Here, I present novel data using next generation sequencing (NGS) to explore the impact of incubation temperature on the skin microbiota of leatherback neonates at emergence and at 3–4 weeks of age.

Methods

Neonate selection and husbandry

The leatherback neonates involved in this study originated from naturally oviposited nests in Juno Beach and Boca Raton, Florida. Neonates were collected during the 2021, 2022, and 2023 hatching seasons (May–August). Throughout each season nests were routinely monitored for emergence. Upon emergence all neonates were transported to the Florida Atlantic University (FAU) Marine Laboratory in Boca Raton, Florida. All neonates were visually examined within 12 hours of emergence. Up to 5 neonates from each nest were selected for entry into the research colony at the FAU Marine Laboratory. All of those selected for entrance into the colony appeared clinically normal (i.e., active, alert, and had no obvious external abnormalities). All neonates were housed and cared for as specified in Kuschke et al., 2023.

Neonate sample collection

Skin swabs were collected from neonates entering the FAU research colony on the day of emergence and again at 3–4 weeks of age. Sample collection followed the same procedure at both time points. Each neonate was rinsed with 50 mL of sterile nano-pure water four times. Due

to their small size, a single sterile swab (rayon or polyester) was used to sample the animal's whole integument including the head, carapace, plastron and all four limbs. Each region was swabbed twice with the single swab. Swabbing was repeated in duplicate for each neonate. Each swab was placed into a sterile vial and was stored at -80°C until DNA extraction.

DNA extractions

All DNA extractions were performed using the DNeasy® PowerSoil® Pro Kit (QIAGEN®) according to manufacturer's protocol with the following modifications. To start swabs were placed into the PowerBead Pro Tubes with 800 µl of CD1 solution and left to sit for 5 minutes. PowerBead Pro Tubes were then vortexed for 10 minutes with the swabs still in the tubes. The supernatant was removed from the PowerBead Pro Tube from around the swab (i.e., the swab was still within the tube). The manufacturer's protocol was then followed until the final step which had the following modification. Solution C6 was left to sit on the center of the white filter membrane for 5 minutes prior to centrifuging. All DNA was stored at -80°C until sequencing.

Next-generation sequencing

All DNA was submitted to the University of Tennessee Genomics Core for polymerase chain reaction (PCR) and NGS. Next-generation sequencing was performed using the V3-V4 region of the 16S rRNA gene with forward primer 5' CCTACGGGNGGCWGCAG 3' and reverse 5' GACTACHVGGGTATCTAATCC 3' on the Illumina MiSeq (Fadrosh et al., 2014).

Sequence processing

Sequence data were analyzed using the DADA2 pipeline (v1. 18.0; Callahan et al., 2016; Bailly et al., 2022) in RStudio (v4.3.1; R Core Team 2023). Taxonomy was assigned using the IDTAXA algorithm in the DECIPHER package (Wright 2016; Murali et al., 2018). Samples with low numbers of reads (i.e., <10,000) were removed prior to statistical analysis.

Incubation temperature collection and analysis

Temperature data loggers were placed into all nests within 12 hours of oviposition. Temperatures were recorded throughout the entirety of incubation at 15- or 30- minute intervals. The average incubation temperature across all nests was ~31°C. To assess the amount of time that each nest spent above this average, incubation temperature data from each nest were converted into the percent of time spent at or above 31°C. The average percent of incubation spent at or above 31°C across all nests was ~40%. As such, 40% was the chosen cut off point to classify each nest with incubation temperatures as either cooler or hot. Spending <40% of incubation at or above 31°C was considered cooler and spending ≥40% of incubation at or above 31°C was considered hot.

Statistical analysis

All data analyses were performed using Rstudio (v4.3.1; R Core Team 2023). Packages used included 'phyloseq', 'vegan', 'devtools', 'microbiome', and 'ggplot' (McMurdie and Holmes 2023; Oksanen et al., 2022; Wickham et al., 2022; Lahti and Shetty 2019; Wickham 2016). Alpha diversity was explored using Observed OTUs, Shannon index, Chao1 estimates,

and Inverse Simpson index. Paired comparisons between temperature groups were made using a pairwise Wilcoxon rank sum test with Holm p-values that were adjusted for multiple comparisons. Beta diversity was explored using principal co-ordinate analysis (PCoA) (Bray-Curtis) and Adonis tests to compare between cooler and hot temperature groups.

Results

Neonates at emergence

Across all three years, 178 neonatal leatherbacks were sampled at emergence (Table 4.1). The three most abundant bacterial phyla, at emergence, were Proteobacteria, Bacteroidota, and Actinobacteria (Table 4.2, Figure 4.1). Two phyla were present in similar amounts on neonates that emerged from both hot and cooler nests, though Actinobacteria were two times more prominent on neonates from cooler nests compared to those from hot nests. The most predominant bacterial families found at emergence were Xanthomonadaceae, Moraxellaceae, and Weeksellaceae (Table 4.3, Figure 4.2). The order of predominance of these families differed between neonates that emerged from cooler versus hot nests. The predominant bacterial family on the skin of neonates that emerged from cooler nests was Moraxellaceae. On the skin of neonates that emerged from hot nests, the predominant bacterial family was Xanthomonadaceae. Analysis of alpha diversity revealed no significant differences between neonates that emerged from cooler and hot nests as measured by Observed OTUs, Chao1 estimates, Shannon, and Inverse indices. However, clustering patterns of incubation temperatures were observed during analysis of beta diversity ($R^2 = 0.03$, $p = 0.001$; Figure 4.3).

Neonates at 3–4 weeks of age

Across all years, 140 leatherback neonates were sampled at 3–4 weeks of age (Table 4.1). Abundant bacterial phyla at 3–4 weeks of age included Proteobacteria, Bacteroidota, and Actinobacteriota (Table 4.4, Figure 4.4). Relative abundance (RA) of each was similar when compared between incubation temperature groups (i.e., cooler and hot). Abundant bacterial families included Flavobacteriacea, Rhodobacteracea, Enterobacteriacae, and Xanthomonadaceae (Table 4.5, Figure 4.5). Relative abundance of each family was similar except for Enterobacteriaceae, which was notably higher on neonates from cooler nests than those from hot nests with relative abundances of 12.8 and 7.8 respectively. Analysis of alpha diversity revealed no significant differences between neonates that emerged from cooler and hot nests as measured by Observed OTUs, Chao1 estimates, Shannon, and Inverse indices. However, clustering patterns of incubation temperatures were observed during analysis of beta diversity ($R^2 = 0.03$, $p = 0.001$; Figure 4.6).

Discussion

Neonates at emergence

Differences existed in the most prominent bacterial families between neonates that incubated in cooler versus hot nests. In neonates that emerged from cooler nests, the most predominant bacterial family was Moraxellaceae. This differed from the most predominant bacterial family on neonates that emerged from hot nests, which was Xanthomonadaceae. These

shifts did not significantly impact the diversity on the individual as there were no significant differences in the alpha diversity between groups. However, beta diversity was significantly impacted by these shifts. Clustering patterns, though overlapping, were significantly different between neonates that incubated in cooler versus hot nests. This indicates that the bacterial communities on the skin of neonates that incubated in hot nests are truly different from those that incubated in cooler nests. This statistically significant difference suggests that increasing incubation temperatures are correlated with alterations in the bacterial community on the skin of leatherback neonates at emergence. However, the nest environment is dynamic and can be impacted by a number of other factors such as location on the beach, inundation events, and other anthropogenic influences. While beyond the scope of this study, additional factors impacting the nest environment and possibly skin microbiota should be investigated in the future.

Bacteria within the family Moraxellaceae are commonly found in nature and have been identified in soil, plants, water, food, and on the skin of humans and animals (Teixeria and Merquior 2014; Hu et al., 2022; Kanjer et al., 2022). While some genera within this family can cause clinical infections, most are considered to have no clinical importance (Teixeria and Merquior 2014). Consequently, this family of bacteria is likely a part of the normal community found on the skin of leatherback sea turtles at emergence. The RA of Moraxellaceae was lower in neonates that incubated in hot nests. It is possible that increased or prolonged heat within the nest caused this decrease. A study of the rumen microbes in Hanwoo steer reported a decrease in Moraxellacea after prolonged exposure to heat (Baek et al., 2020). However, this decrease in RA could also be relative to the increase in RA of Xanthomonadaceae observed in turtles that emerged from hot nests.

Many bacteria within the family Xanthomonadaceae, previously known as Lysobacteraceae, are commonly associated with plants (Saddler and Bradbury 2005; Puopolo et al., 2014; Naushad et al., 2015; Assis et al., 2017; Giovanardi et al., 2018; Cutiño-Jiménez et al., 2020). However, bacteria within this family have also been found on the skin of humans and animals and it's likely a part of the normal skin microbiota (Walke et al., 2015b; Bletz et al., 2017; Zeng et al., 2017; Mutnale et a., 2021). The cause of the increase in RA of this family of bacteria observed on neonates that incubated in hot nests is not currently known. However, this bacterial family contains the genus *Lysobacter,* which is known to produce protective lytic enzymes and antifungal agents (Reichenbach 2006; Chen et la., 2017; Chen et al., 2018; Zhao et al., 2019). In 2020, the fungal disease, sea turtle egg fusariosis (STEF) was identified as an emerging disease in sea turtles, resulting in decreased hatching success in all species (Sarmiento-Ramírez et al., 2014; Gleason et la., 2020). Climate change is a driving factor worsening the problem of fungal infections in humans and animals (Garcia-Solache and Casadevall, 2010; Casadevall, 2018; One Health, 2019). Hot nests may be experiencing shifts within the sand microbiota such as increased presence of *Fusarium* spp. It is possible that the increase in Xanthomonadaceae seen on turtles that incubated in hot nests is a biological response or possibly compensation for exposure to *Fusarium* spp. The causative agents of STEF (*Fusarium* spp.) are not only impacting sea turtle eggs but have also been reported to cause mycotic dermatitis in leatherback sea turtle neonates (Kuschke et al., 2023). Thus, further investigation into Xanthhomonadaceae as a potential means to combat the negative impacts of STEF in both leatherback nests and neonates is recommended.

Neonates at 3–4 weeks

Major shifts in the most predominant phyla and families were not seen when comparing neonates that incubated in cooler versus hot nests at 3–4 weeks of age. Though, there was notably less RA of the family Enterobacteriaceae in neonates that incubated in hot nests. Enterobacteriaceae is a bacterial family that has been previously isolated from the skin of amphibians and mucosal surfaces of sea turtles (Beltz et al., 2017; Passos et al., 2018; Kuschke, 2022). It is likely a part of the normal microbiota on the skin of sea turtles; however, the significance of the decreased abundance of this bacterial family in neonates that incubated in hot nests is unclear and requires further investigation.

While no significant differences were observed between measures of alpha diversity, beta diversity was significantly different between neonates that incubated in cooler versus hot nests. This indicates that the bacterial communities on the skin of neonates that incubated in hot nests are truly different from those that incubated in cooler nests even 3–4 weeks after emergence. This suggests that increasing incubation temperatures have long-term impacts on leatherback skin microbiota. It is important to note that the leatherback neonates included in this study were temporarily maintained in a research facility under controlled water and environmental conditions (Kuschke et al., 2023). Unfortunately, in the wild leatherback neonates would also be combatting increasing ocean temperatures (Brierley and Kingsford 2009; NMFS and USFWS, 2020). Previous research has shown that increased water temperatures alter the skin and gut microbiota in fish (Gosh et al., 2022; Yang et al., 2022a). Yang et al., 2022a reported a significant disruption in diversity and abundance of bacteria correlated with increasing temperatures. Higher water temperatures also increased the presence of potential pathogenic bacteria and decreased the presence of known probiotics (Yang et al., 2022a). Ultimately, increased water temperatures result in dysbiosis of the skin microbiota (Gosh et al., 2022; Yang et al., 2022a), which is associated with negative impacts on health and often results in the development of disease (De Pessemier et al., 2021). If these trends are also true for leatherback neonates experiencing rising ocean temperatures, it would suggest that wild neonates that emerge from hot nests are unlikely to recover from the alteration in their skin microbiota seen in this study, and ultimately, the microbial shifts, or dysbiosis, would be expected to have negative impacts on health and survival.

Future studies investigating the impact of increasing temperatures (beach and ocean) on neonatal leatherback skin microbiota will similarly need to be conducted within a controlled laboratory environment, as wild neonatal encounters are extremely rare. Nevertheless, opportunistic sampling of wild adults, juveniles, and neonates should also be explored. Such samples would be beneficial and aid in establishing a baseline skin microbiota in wild populations and help to further elucidate the impacts of increasing temperatures on the skin microbiota of leatherback sea turtles.

Conclusion

This study is the first to report the impacts of increasing incubation temperatures on neonatal leatherback sea turtle skin microbiota. These data revealed that higher incubation temperatures are altering the skin microbiota of leatherback neonates at emergence. Furthermore, differences in the microbial communities on the skin of neonates from cooler nests compared to those from hot nests remain for up to 3–4 weeks of age. Additional factors influencing the

microbial growth on the skin of neonates in the nest such as fungal pathogens, water inundation events, and nest location should be explored. Moreover, investigations into the long-term impact of rising beach and ocean temperatures on the skin microbiota at all life-stage classes of the leatherback should be investigated. Understanding host-microbial and environment relationships, the factors that influence them, and the long-term impact that they have on health are some of the next critical steps in leatherback conservation and management.

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Appendix

Table 4.1 Summary of sequencing results

	Turtles sampled	**Number of samples submitted for PCR and NGS**	**Samples used for analysis**	**Total reads**	**Average reads**
Neonates at emergence	178	172	151	7,503,179	49,690
Neonates at 3-4 weeks	140	135	126	7,337,226	58,232

Table 4.2 Summary of relative abundance of top 10 most abundant bacterial phyla in neonates at emergence between incubation temperatures. *Cooler: <40% of incubation ≥31°C; Hot: >40% of incubation ≥31°C*

Phylum	**Cooler**	**Hot**
Proteobacteria	64.1	62
Bacteroidetes	24.2	28.1
Actinobacteria	9	4.5
Bdellovibrionota	1.2	3.8
Firmicutes	0.76	0.77
Verrucomicrobiota	0.19	0.40
Plantomycetota	0.09	0.03
Patescibacteria	0.32	0.23
Myxococcota	0.11	0.02
Chloroflexi	0.05	0.01

Table 4.3 Summary of relative abundance of top 10 most abundant bacterial families in neonates at emergence between incubation temperatures. *Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

Family	**Cooler**	**Hot**
Xanthomonadaceae	11.3	17.0
Moraxellaceae	14.8	11.8
Weeksellaceae	9.2	12.3
Pseudomonadaceae	8.0	10.1
Alcaligenaceae	7.2	5.4
Flavobacteriaceae	7.6	3.9
Chitinophagaceae	1.5	4.6
Nocardiaceae	5.2	2.3
Rhizobiaceae	5.9	5.2
Sphingobacteriaceae	4.7	5.7

Table 4.4 Summary of relative abundance of top 10 most abundant bacterial phyla in neonates at 3–4 weeks between incubation temperatures. *Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

Phyla	**Cooler**	**Hot**
Proteobacteria	67.5	67.5
Bacteroidetes	25.0	22
Actinobacteria	4.0	5.4
Firmicutes	1.5	3.2
Planctomycetota	1.1	1.2
Bdellovibrionota	0.41	0.16
Myxococcota	0.21	0.13
Patescibacteria	0.06	0.26
Verrucomicrobiota	0.05	0.11
Campilobacterota	0.13	0.0001

Table 4.5 Summary of relative abundance of top 10 most abundant bacterial families in neonates at 3–4 weeks between incubation temperatures. *Cooler <40% of incubation ≥31 °C*; *Hot >40% of incubation ≥31 °C*

Family	**Cooler**	**Hot**
Flavobacteriaceae	21.4	20.6
Rhodobacteraceae	14.3	12.0
Enterobacteriaceae	12.8	7.8
Xanthomonadaceae	7.4	6.5
Vibrionaceae	4.2	5.8
Alteromonadaceae	3.2	6.6
Pseudomonadaceae	4.1	5.2
Porticoccaceae	3.3	4.7
Norcardiaceae	3.2	4.2
Rhizobiaceae	2.3	2.4

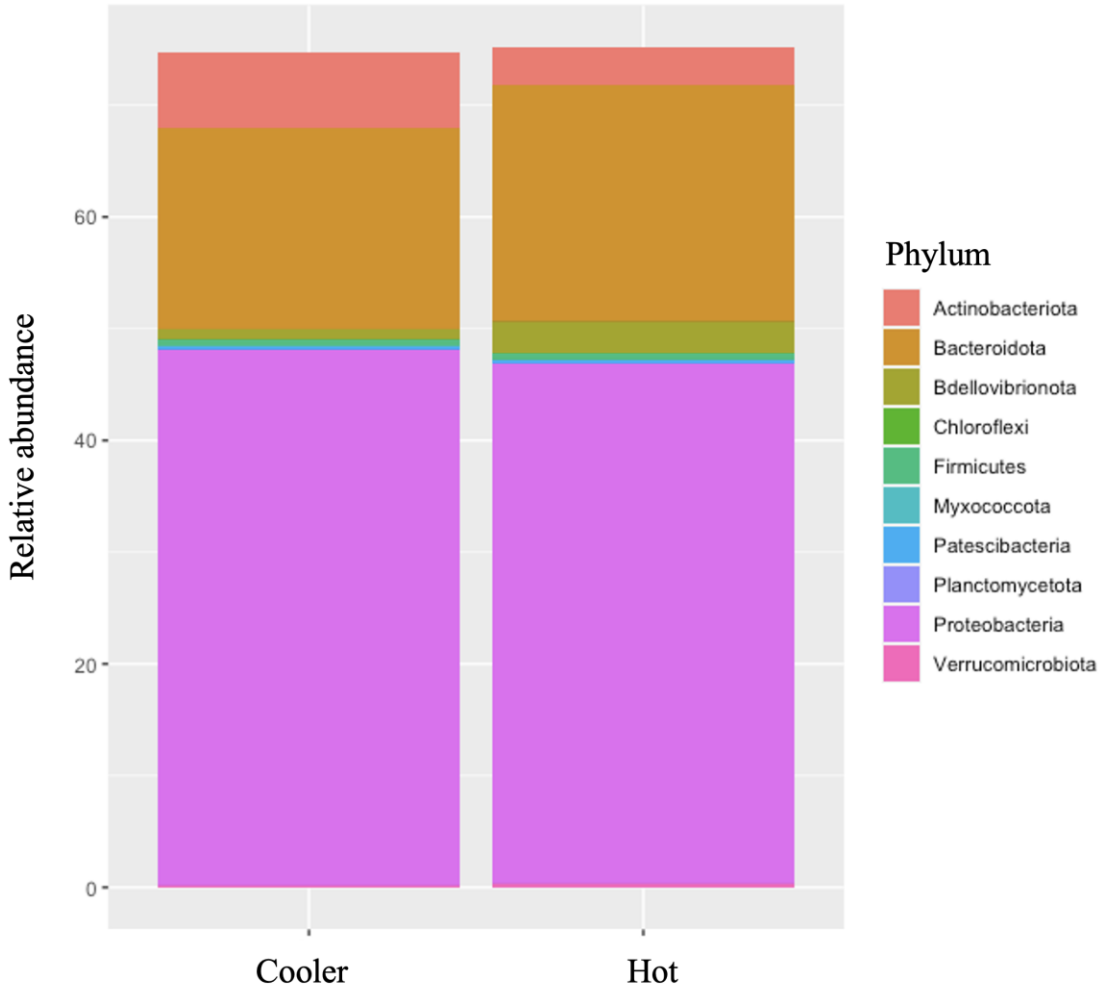


Figure 4.1 Summary of relative abundance of top 10 most abundant bacterial phyla in neonates at emergence between incubation temperatures. Note that the relative abundance of phyla are similar. *Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

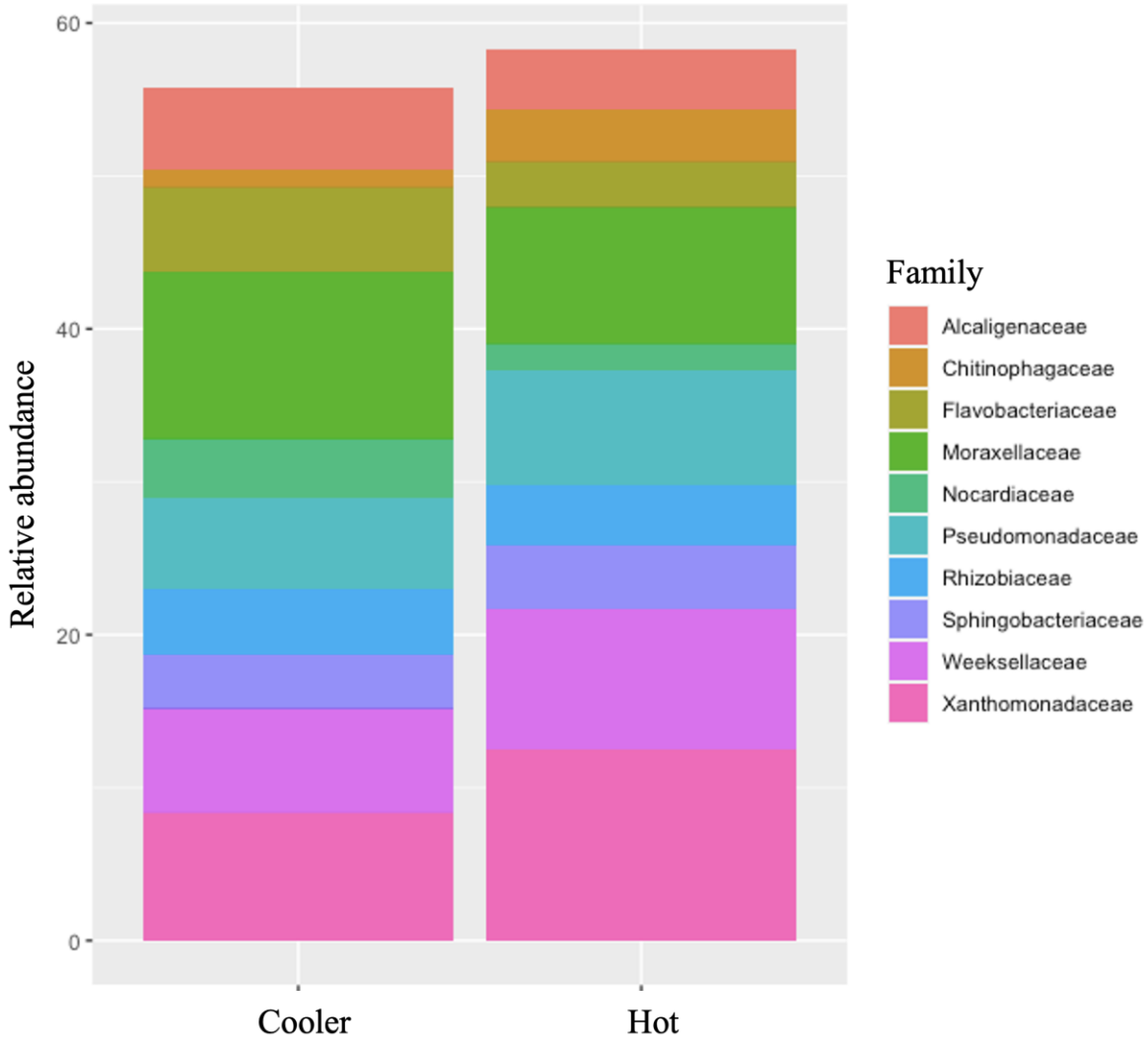


Figure 4.2 Summary of relative abundance of top 10 most abundant bacterial families in neonates at emergence between incubation temperatures. Note that the families are similar between temperature groups. *Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

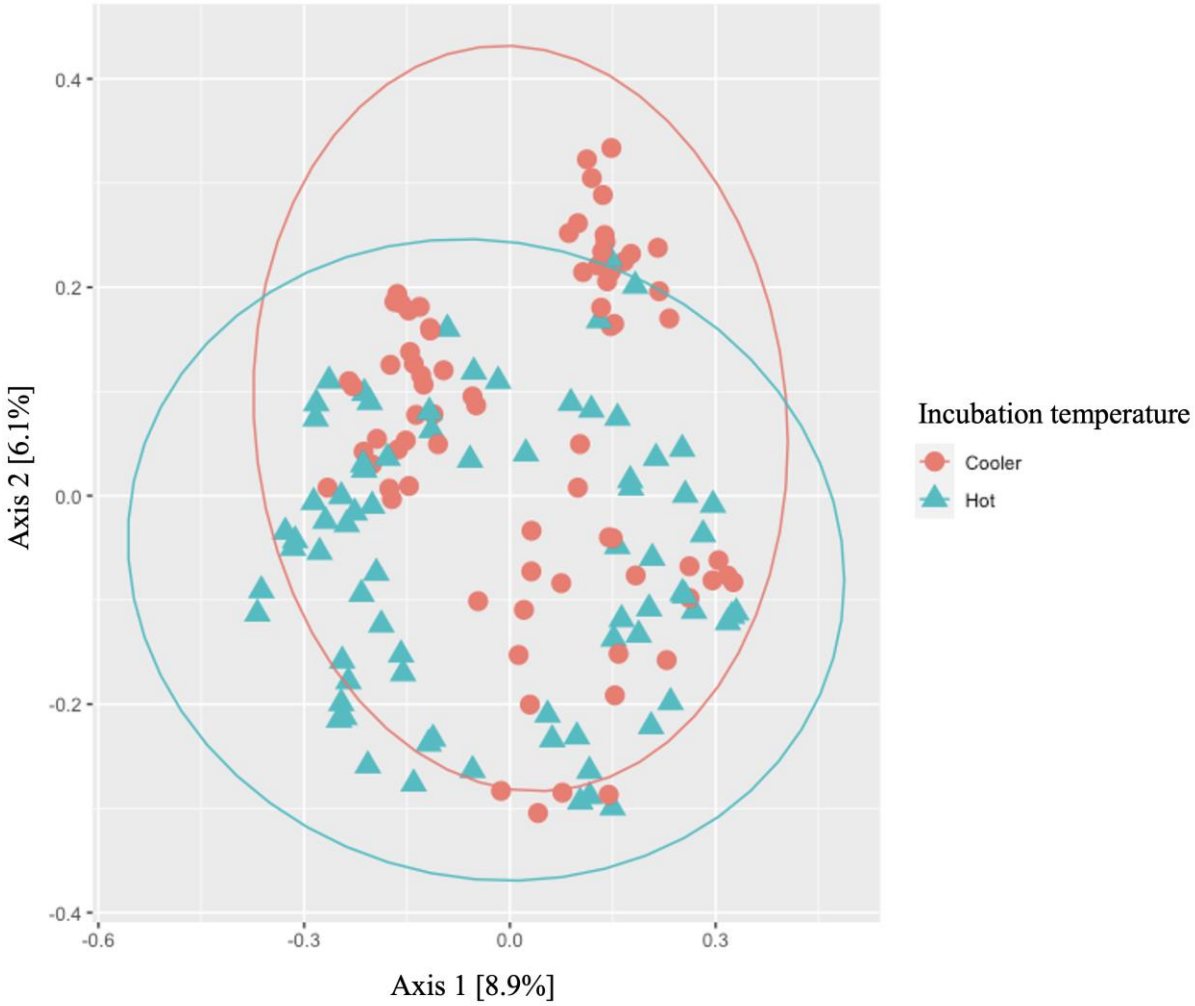


Figure 4.3 Beta diversity expressed using a principal coordinate analysis (PCoA) of Bray-Curtis distance showing clustering patterns between neonates at emergence that incubated in hot versus cooler nests. Significant differences in microbial communities existed between temperature groups ($R^2 = 0.03$, $p = .001$). *Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

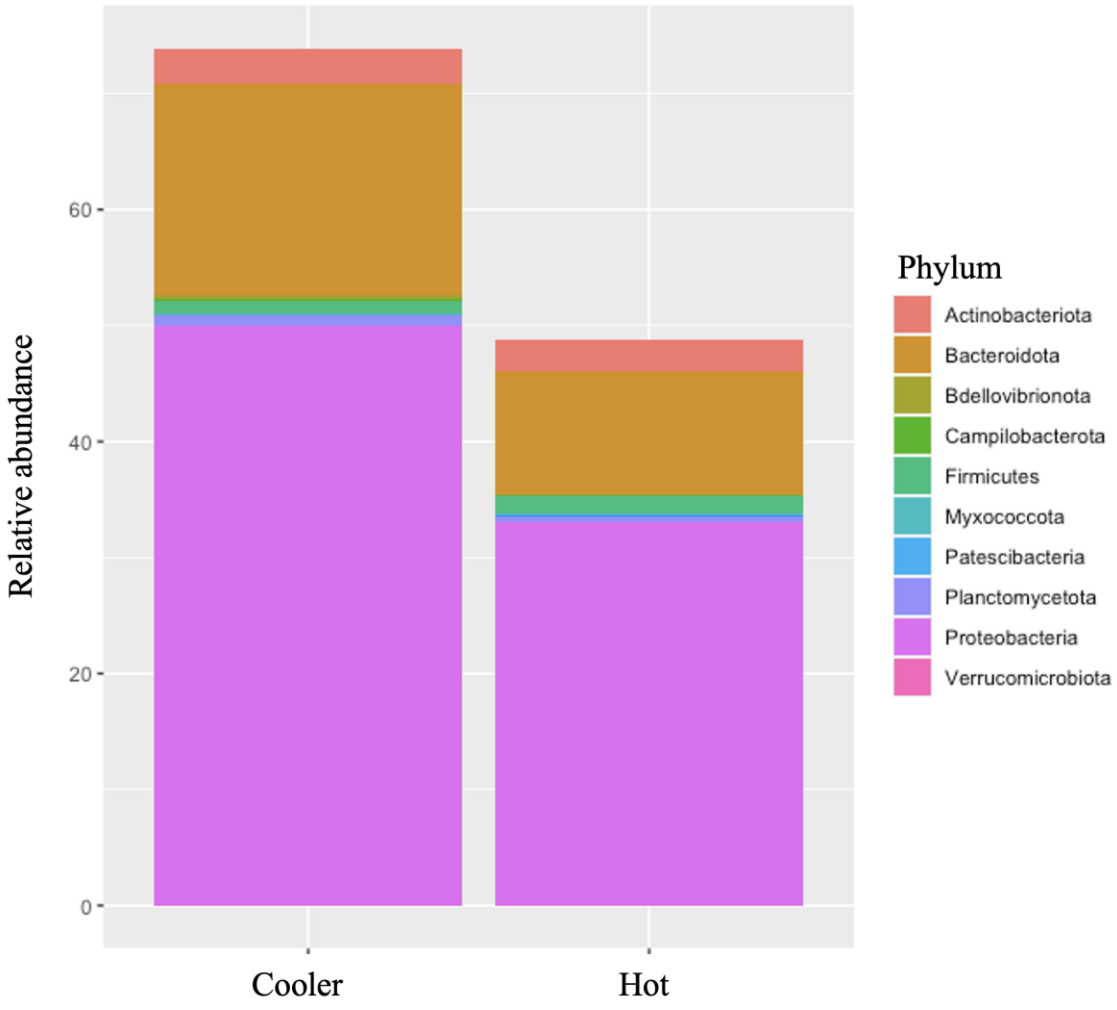


Figure 4.4 Summary of relative abundance of top 10 most abundant bacterial phyla in neonates at 3–4 weeks between incubation temperatures. Relative abundance of each phylum was similar across temperature groups. *Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

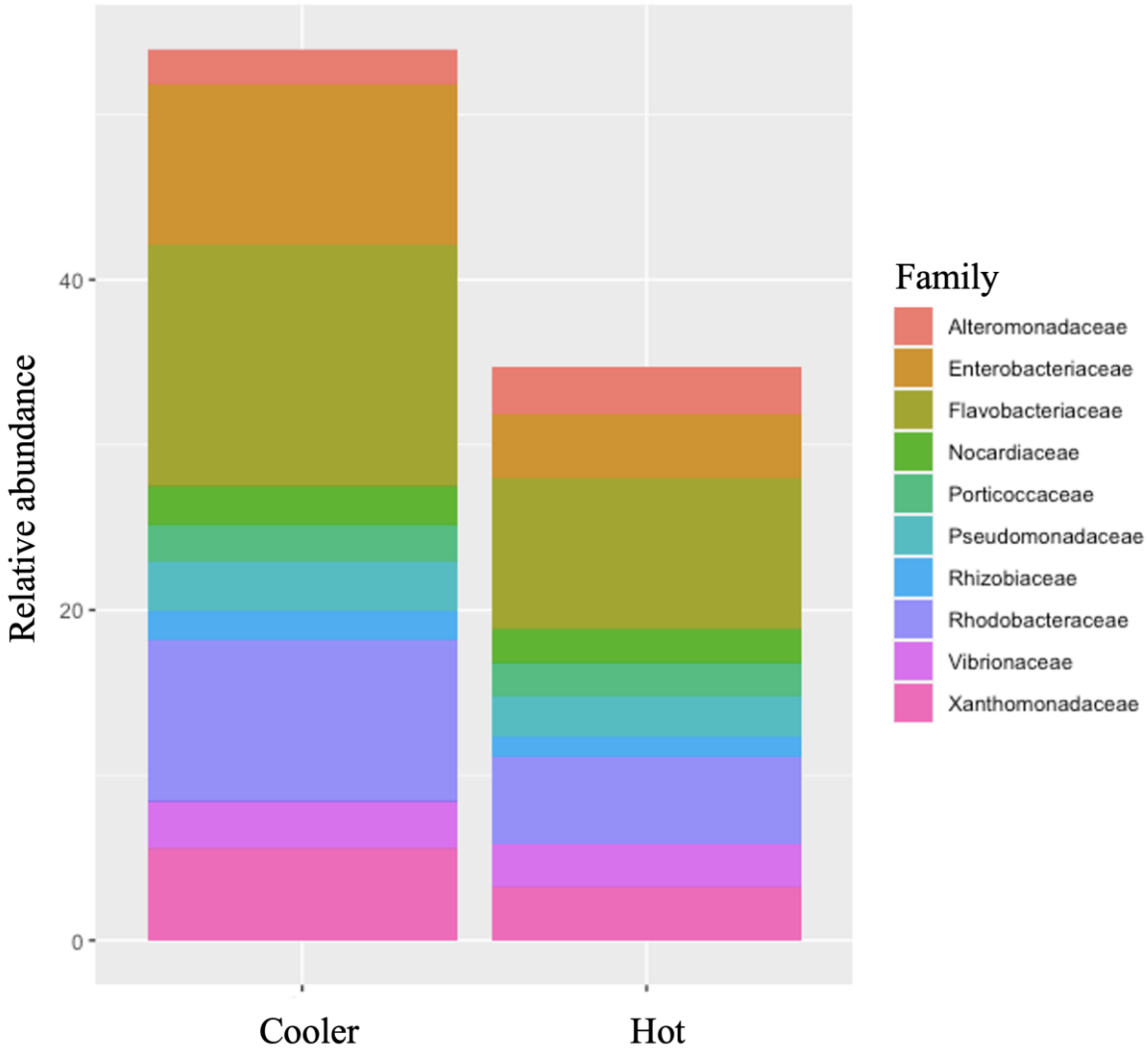


Figure 4.5 Relative abundance of top 10 most abundant bacterial families in neonates at 3–4 weeks between incubation temperatures. The relative abundance of each family was similar between the temperature groups except for Enterobacteriaceae, which was notably higher on neonates from cooler nests. *Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

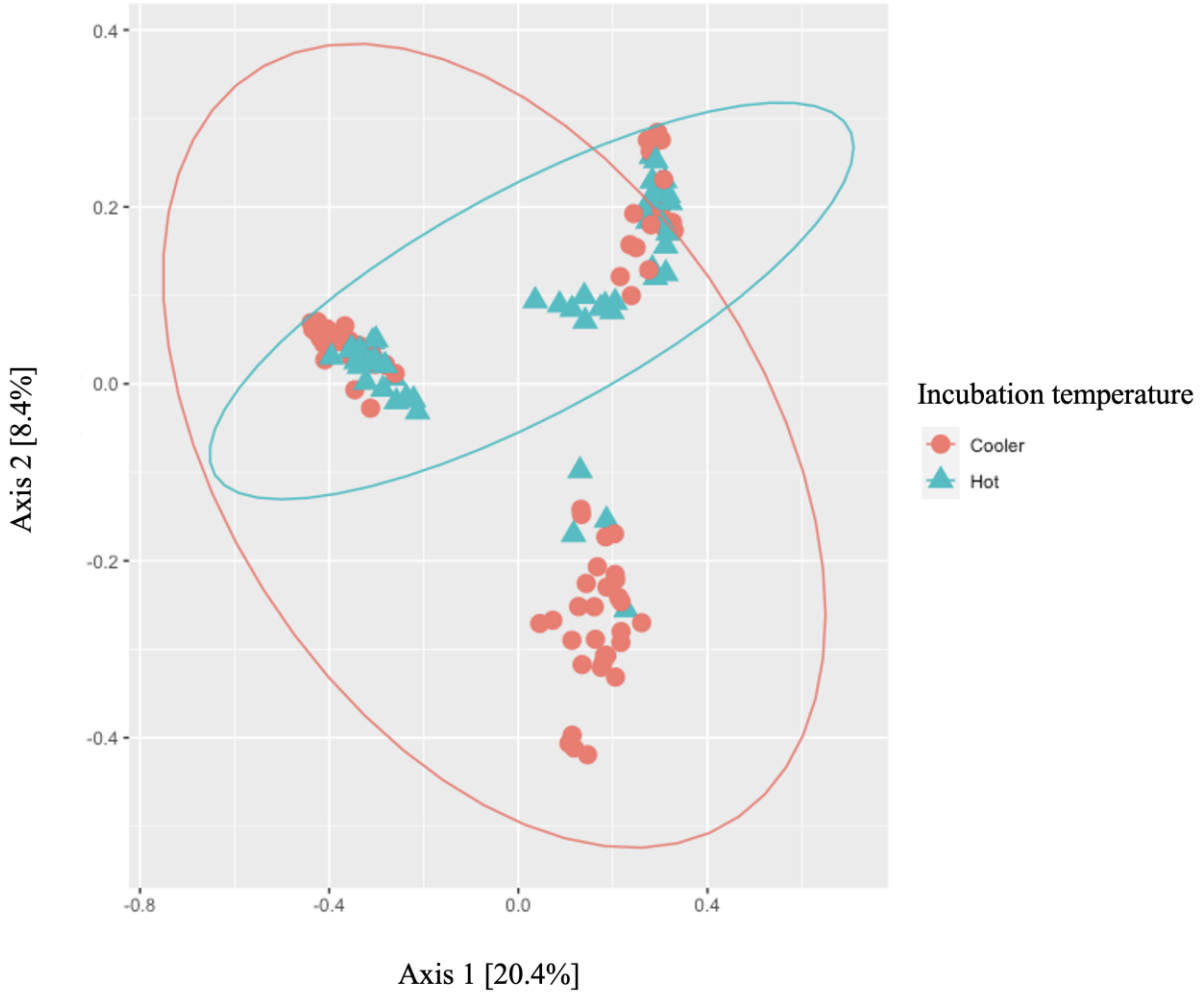


Figure 4.6 Beta diversity expressed using a principal coordinate analysis (PCoA) of Bray-Curtis distance showing clustering patterns between neonates at 3–4 weeks that incubated in hot versus cooler nests. The three major clusters of samples spanned ~-0.4, another just above 0 and the just above 0.2 along Axis 1. The majority of the hot samples occurred along the same Axis 1 ranges. Most tended to cluster at or above 0 along Axis 0.2. The significance of the 3 clusters of points is unclear. Nevertheless, significant differences in microbial communities existed between temperature groups ($R^2 = 0.03$, $p = .001$). *Cooler: <40% of incubation ≥31 °C; Hot: >40% of incubation ≥31 °C*

CONCLUSION

The causes of the conservation status of leatherback sea turtles are multifactorial, however one of the driving factors is climate change (NMFS and US FWS, 2020; IUCN, 2023). The consequences of climate change are affecting leatherbacks at multiple points during their life cycle and resulting in negative impacts on health (NMFS and US FWS, 2020; IUCN, 2023). Currently, large efforts are aimed at enacting appropriate conservation measures to combat climate change (Cox et al., 2007; Fuentes et al., 2012; Jualaong et al., 2021; Kanghae et al., 2024). Unfortunately, many of these efforts are currently being thwarted by a fundamental lack of knowledge of the species (Jones et al., 2000; Jones et al., 2011; Hamann et al., 2010; Fuentes et al., 2011). Large questions remain regarding what defines a healthy leatherback and how climate change is impacting them (Hamann et al., 2010). The studies presented in this work aim to start filling some of these large data gaps – namely identifying unique measures of health in leatherback sea turtles and determining how increasing incubation temperatures are impacting them.

Blood analytes are diagnostic tools often used to assess the health of turtles (Cray and Tatum 1998; Zaias and Cray 2002; Melillo 2013; Stacy and Innis 2017; Stacy et al., 2017; Deem and Harris 2017, Fleming et al., 2020; Cray, 2021; Perrault et al., 2022). However, species and age class-specific reference intervals are necessary for appropriate interpretation of the values obtained (Stacy and Innis 2017; Stacy et al., 2017; Deem and Harris 2017). Protein electrophoresis (EPH) is one such diagnostic tool, and its use has been expanding in veterinary medicine with our growing knowledge of its applications (Cray and Tatum 1998; Zaias and Cray 2002; Melillo 2013; Cray 2021). Unfortunately, current studies of EPH in leatherbacks are limited to nesting females (Deem et al., 2006; Perrault et al., 2014; Stacy et al., 2019). Thus, I am were interested in establishing reference intervals for EPH and other key blood analytes in neonatal leatherbacks and observing if values shifted in response to incubation temperature. I found that higher incubation temperatures were correlated with shifts in blood analytes that indicated both dehydration at emergence and inflammation at both emergence and 3-4 weeks of age. The continued inflammation observed in neonates at 3-4 weeks of age suggests that incubation temperatures have long-term impacts on health. Thus, future studies should further investigate the inflammation present and its impact on neonatal success.

The microbiota on and within animals is now known to play a key role in host health and can serve as an indicator of it (Sommer and Backhed 2013; Selosse et al., 2014; Colston and Jackson 2016; Walke and Belden 2016; Simon et al., 2019). Consequently, our next goal was to identify and characterize the skin microbiota in leatherback sea turtles. Previous studies have investigated the microbiota located on other regions of sea turtles, but research on the skin microbiota of sea turtles is lacking (Kuschke, 2022). Due to the leatherback's unique skin composition, its skin microbiota is of great interest (Kuschke, 2022). Here I established baseline skin microbiota for multiple life-stages of leatherback sea turtles and identified significant differences in bacterial communities existed based on life-stage classes. Additionally, we examined the impact of increasing incubation temperatures on the skin microbiota in neonates at emergence and 3–4 weeks of age. Significant shifts in skin microbiota, correlated with higher incubation temperatures, were observed at both time points. At emergence a higher level of a potentially protective bacterial family was observed in neonates that emerged from hot nests when compared to cooler nests. At 3–4 weeks major shifts in the dominant bacterial phyla and families were not found, yet communities still differed significantly between temperature groups.

In depth investigations into the role of each microorganism present within the skin microbial community was beyond the scope of this work. However, given the significant differences observed between temperature groups, further investigations are warranted. The significance of shifts within the bacterial communities should be studied to gain further understanding of the host-microbiota interactions occurring and the potential impacts these shifts have on long- and short-term health.

Overall, the studies presented here begin to answer two of the meta-questions posed by Hamann et al. (2010): "What constitutes a healthy turtle?" and "What will be the impacts from climate change on sea turtles . . .?". The baseline data presented here can serve as a means to measure the health of leatherback sea turtles. Additionally, our investigations into the impact of climate change on health revealed that not only are increasing incubation temperatures impacting leatherback neonates at emergence, but alterations in health parameters remain for up to 3-4 weeks. This suggests that climate change is negatively impacting health and thus the potential survival of leatherback neonates into their first month of life. Accordingly, this work provides a partial explanation for decreased survival in hatchlings incubated at higher temperatures. Though data gaps still persist, the data herein should be used as baseline information to begin developing management strategies aimed at mitigating the effects of elevated incubation temperatures on leatherback sea turtles. Future studies should further investigate the inflammation observed in neonates via additional diagnostic tests such as white blood cell counts or other acute-phase proteins. Additionally, host-microbiota interactions should be explored as potential means of protection against growing environmental threats, particularly rising temperatures.

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VITA

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