Impacts of host and environment on Elasmobranch microbiomes

By

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ABSTRACT

Elasmobranchs are critical for regulating prey populations, structuring coastal habitats, and maintaining biodiversity. Unfortunately, Elasmobranchs are susceptible to overfishing, climate change, and pollution leading to global declines in their populations. Microbiomes are important for Elasmobranch health and ecology, but few host species' microbiomes have been described. My thesis expands current knowledge of Elasmobranch microbial ecology by describing novel relationships between hosts and their microbiomes. I explore host and environmental influences on wild Elasmobranch microbiomes using a combination of shotgun metagenomic sequencing, High Performance Liquid Chromatography (HPLC), and long read sequencing of bacterial isolates.

I explore the skin, gill, and cloaca microbiomes of two hosts (*Myliobatis tenuicaudatus* and *Heterodontus portusjacksoni*) living in the same environment. Both hosts have unique microbiomes, indicating host shapes taxonomic communities at all body sites. Microbiome functions were also unique between hosts, except for skin, indicating skin microbes are adapted to host and environment. A Metagenome Assembled Genome (MAG) of *Photobacterium damselae*, with novel prophage sequences was reconstructed from *M. tenuicaudatus. Photobacterium damselae* is consistently found in shark gut samples and this MAG is closely related to *P. damselae* originating from other Elasmobranchs.

I compare skin microbiomes of four juvenile host species across two sampling events to determine environmental influence on the microbiome. Juvenile host microbiomes were similar, but microbiomes were different between sampling events. Juvenile Elasmobranchs are influenced by the environment more than adults. To describe environmental influence across locations, I compare microbiomes of two species (*Urobatis halleri* and *Myliobatis californica*) in San Diego and Los Angeles California. *Myliobatis californica* had highly variable skin microbiomes that were similar across locations, likely due to the thick epidermal mucus layer. Despite their shared environment, hosts maintain distinct microbial communities from each other, and the water column indicating host is driving microbial community diversity.

To explore the mechanisms of microbiome selectivity on Elasmobranchs, I quantified the monosaccharide composition of mucus from four host species. Hosts had unique mucus monosaccharide composition. Host microbiomes had carbohydrate metabolism genes to utilize mucus monosaccharides. I described for the first time, Elasmobranch microbial genes selected by host mucus composition. I also describe genomic adaptations of bacteria isolated from white sharks (*Carcharodon carcharias*), including mobile genetic elements and plasmids.

Finally, I compare microbiomes of sixteen host species from South Australia and California. I compare metagenome similarity across and within host clades. Within the genus *Myliobatis* two species *(Myliobatis tenuicaudatus* and *Myliobatis californica)* have the most similar microbiomes, even when sampled from across the world indicating phylosymbiosis is strongest within a clade.

Overall, I described host (mucus composition, evolutionary history) and environmental (habitat, season) factors that influence Elasmobranch microbiomes. I describe the microbiome of an additional eight Elasmobranch host species from South Australia, almost doubling the number of Elasmobranch host microbiomes described previously. I contribute novel insights into Elasmobranch microbiome functions in the gill and cloaca and make the first link between microbiome and host mucus in Elasmobranchs. These findings will inform future efforts to describe host-microbiome relationships in Elasmobranchs and how this relationship will change with anthropogenic stress.

DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

4. has been completed without the use of generative artificial intelligence tools.

Signed Emma Nicole Kerr

Date August 21, 2025

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ACHIEVEMENTS

Publications

Publications written and submitted during candidature are included below.

Kerr, E.N., B. Papudeshi, M. Haggerty, N. Wild, A.Z. Goodman, L.F.O. Lima, R.D. Hesse, A. Skye, V. Mallawaarachchi, S. Johri, S. Parker, E.A. Dinsdale. 2023. Stingray epidermal microbiomes are species-specific with local adaptations. *Front. Microbiology*. 14:1031711. doi: 10.3389/fmicb.2023.1031711

Kerr, E.N., R.D. Hesse, J.A.P. Carlson-Jones, B. Nalagampalli Papudeshi, P.A. Butcher, M.P. Doane, E.A. Dinsdale. 2025. Draft genomes of five bacteria isolated from *Carcharodon carcharias* (white shark) and *Carcharhinus brachyurus* (bronze whaler shark). *Microbial Resource Announcements*. https://doi.org/10.1128/mra.00226-25

Kerr, E.N., L. Yu, R.D. Hesse, C.N. Roberts, V. Bulone, L. Meyer, R.A. Edwards, M.P. Doane, E.A Dinsdale. In Review. Interactions of mucus monosaccharides and the epidermal microbiome in four benthic Elasmobranchs. Environmental Microbiology Reports.

Goodman, A.Z. B. Papudeshi, M. Mora, **E. N. Kerr,** M. Torres, J. N. Moffatt, L. F.O. Lima, I. R. Niesman, I. Y. Moreno, M. P. Doane, E. A. Dinsdale. 2024. Elasmobranchs Exhibit Species-Specific Epidermal Microbiomes Guided by Denticle Topography. BioRxiv. bioRxiv 2024.04.05.588334; doi: https://doi.org/10.1101/2024.04.05.588334

Butcher P. ... **E.N. Kerr**... et al., In Review. Conserve, cull, compromise — the white shark conundrum. *Wildlife Reports*.

Grants & Awards

Grants and awards received during candidature are included below in chronological order.

Flinders University Student Association Development Grant: $800 AUD
Royal Society of South Australia Small Research Grant: $2500 AUD
National Science Week Outreach Grant: $2400 AUD
Flinders University Student Association Women in STEM Grant: $1500 AUD
Soroptimist International Travel Award: $500 AUD
College of Science and Engineering Higher Degree by Research Travel Award: $500 AUD
Flinders University Student Association Development Grant $750 AUD
South Australia/Northern Territory Australian Society for Microbiology Finalist $60 AUD
Higher Degree by Research Leadership and Excellence Award $500 AUD
College of Science and Engineering Quarterly Staff Award
Inspiring South Australia Outreach Grant $4,500 AUD

AUTHOR CONTRIBUTIONS

Published Manuscripts

Title: Stingray microbiomes are species specific with local adaptations

Author	Participation
Emma N. Kerr	Microbiome collection, DNA extraction, library preparation, sequencing, bioinformatics, statistics, wrote & edited manuscript
Bhavya Papudeshi	Bioinformatics
Lais F.O. Lima	Facilitated library preparation and sequencing
Amber Skye Natasha Wild	Assisted with MAG analysis
Miranda Haggerty Ryan Hesse Asha Goodman Sophia Parker	Facilitated sample collection
Elizabeth Dinsdale	Supervision, edited manuscript

Title: Draft genomes of five bacteria isolated from *Carcharodon carcharias* (white shark) and *Carcharhinus brachyurus* (bronze whaler shark)

Author	Participation
Emma N. Kerr	Bacterial culture, DNA extraction, library preparation, sequencing, bioinformatics, data analysis, wrote & edited manuscript
Ryan Hesse Paul Butcher	Collected samples
Jessica Carlson-Jones	Assisted with sequencing
Bhavya Papudeshi	Bioinformatics
Mike Doane	Supervision, edited manuscript
Elizabeth Dinsdale	Supervision, co-wrote & edited manuscript

Title: Interactions of mucus monosaccharides and the epidermal microbiome in four benthic Elasmobranchs

Author	Participation
Emma N. Kerr	Collected samples, processed samples for HPLC and DNA sequencing, bioinformatics, data analysis, wrote & edited manuscript
Long Yu, Vincent Bulone	Facilitated sample processing for HPLC
Ryan Hesse Chloe Roberts Lauren Meyer	Collected samples
Rob Edwards	Bioinformatics
Mike Doane	Supervision, edited manuscript
Elizabeth Dinsdale	Supervision, co-wrote & edited manuscript

Manuscripts in Preparation

Title: Slimy vs. Scaly: Identifying microbiome diversity and functions across skin, gill and cloaca in two benthic Elasmobranchs (Chapter 1)

Author	Participation
Emma N. Kerr	Sample collection, DNA, extraction, data analysis, and wrote manuscript
Belinda Martin	Facilitated DNA Extraction
Chloe Roberts	Sample collection
Robert A. Edwards	Bioinformatics
Michael Doane Elizabeth Dinsdale	Supervision, edited manuscript

Title: Juvenile elasmobranch microbiomes are influenced by but distinct from the environment (Chapter 2)

Author	Participation
Emma N. Kerr	Sample collection, DNA extraction, library preparation, sequencing, bioinformatics, data analysis, and wrote & edited manuscript
Ryan Hesse	Collected samples, flow cytometry processing
Robert A. Edwards	Bioinformatics
Michael Doane Elizabeth Dinsdale	Supervision, edited manuscript

Title: Phylosymbiosis is maintained across global benthic Elasmobranch microbiomes (Chapter 6)

Author	Participation
Emma N. Kerr	Sample collection, DNA extraction, library preparation, sequencing, bioinformatics, data analysis, wrote & edited manuscript
Asha Z. Goodman	Sample collection and sequencing
Ryan Hesse Chloe Roberts	Collected samples
Mike Doane	Supervision, edited manuscript
Elizabeth Dinsdale	Supervision, co-wrote & edited manuscript

Appendix Chapters

Title: Elasmobranchs Exhibit Species-Specific Epidermal Microbiomes Guided by Denticle Topography

Author	Participation
Asha Z. Goodman	Sample collection, DNA extraction, library preparation, sequencing, bioinformatics, statistics, wrote and edited manuscript
Emma N. Kerr	Sample collection, DNA extraction, library preparation, edited manuscript
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Lais F.O. Lima	Sequencing
Ingrid R. Niesman Isabel Y. Moreno	Microscopy
Melissa Torres Jennifer Nero Moffatt Michael P. Doane	Sample collection
Elizabeth Dinsdale	Supervision, co-wrote & edited manuscript

Title: Cull, Conserve Compromise, the white shark conundrum

Author	Participation
Paul Butcher	Workshop organization, manuscript writing and editing
Emma N. Kerr	Contributed to the “Cull” section, writing and editing
Other Authors	Contributed to writing and editing

INTRODUCTION

General Introduction

Animal microbiomes are critical for heath (Sehnal et al., 2021), ecosystem function (Wilkins et al., 2019), and evolution (Kolodny et al., 2020). Host microbiomes are influenced by their surrounding environment including specific variables like temperature, pH, or broad factors like habitat or location. Microbiomes are critical for host survival and with increasing pressure from climate change, pollution, and other anthropogenic effects, understanding the influences on host microbiomes is critical for predicting the impact on host survival.

In the marine environment, priorities for microbiome research include understanding how host-microbiome interactions are formed and how the environment influences these interactions, especially under increasingly stressful conditions (Trevathan-Tackett et al., 2019). Microbiomes of marine invertebrates, especially corals and sponges, have received much attention due to their importance as foundation species. Microbiomes associated with marine invertebrates are diverse, complex and vary with extrinsic factors, such as temperature, pH and location (Lima et al., 2020; O'Brien et al., 2019; Thurber et al., 2009; Wilkins et al., 2019). Marine vertebrates have received comparatively less attention and the links between host, microbiome and the environment are largely unresolved or viewed through an aquaculture lens (Apprill, 2017; Legrand et al., 2019). Marine microbiomes particularly in the Southern Hemisphere are underrepresented, with sponge and marine mammal microbiomes being most studied (Ochoa-Sánchez et al., 2023).

Elasmobranchs (sharks, rays, and skates) are a unique clade of vertebrates which evolved over 400 million years ago (Aschliman et al., 2012). They play important roles in coastal ecosystems, structuring marine food webs (Lester et al., 2020) and modifying sediments (O'Shea et al., 2012). Their unique skin structure, immune system, genetic

stability and evolutionary success make them interesting model organisms for exploring host-microbiome interactions. Their critical role in ocean ecosystems make understanding how host-microbiome relationships change with environmental conditions crucial for predicting the microbiome response to climate change, pollution, and other anthropogenic stressors.

Elasmobranch epidermal microbiomes have been explored more than other body sites due to the non-invasive nature and ease of sample collection. Skin microbiomes are distinct from the water column and sediment (Bregman et al., 2023; Doane et al., 2022, Gonçalves e Silva et al., 2020). Shark epidermal microbial communities are different between host species, but sharks share a subset of common shark-associated bacterial taxa including Gammaproteobacteria and Alphaproteobacteria (Doane et al., 2017, 2023; Goodman et al., 2022). Closely related host species have more similar microbiomes, known as phylosymbiosis (Doane et al., 2020). The skin microbiome is still dynamic and can change across time and space. For example, across three sampling events during a four-year period, leopard shark epidermal microbiome taxonomy changed, but microbial functions remained similar (Doane et al., 2022). Evidence for functional redundancy suggests that bacteria are specifically adapted to shark skin, and although taxonomy is flexible, shark skin selects for a specific functional community. Whale shark (*Rhincodon typus*) microbiomes have variable taxonomy across ocean basins, but network architecture was similar, suggesting there are innate features of whale shark skin driving this network structure, but allowing for taxonomic fluctuations that are geographically driven (Doane et al., 2023). Similar patterns emerge in black tip reef sharks (*Carcharhinus melanopterus*) where microbiomes were different across sampling locations (Pogoreutz et al., 2019). Although

skin microbiomes are host-specific, geographic location and seasonal fluctuations are evident.

Investigation of body sites other than skin has occurred in sharks, including the oral, cloaca, gill, wound, and gut microbiome (Black et al., 2021; Storo et al., 2021). Ray microbiome studies have predominantly focused on the skin, but investigations of gill and cloaca microbiomes have been explored (Caballero et al. 2020; Gonçalves e Silva et al. 2020; Kearns et al. 2017; Pinnell et al. 2021). Occasionally interactions with stingrays lead to human injury (Lowe et al. 2007). Efforts to reduce injury to humans by clipping the venomous spine of *Urobatis halleri* individuals was conducted at a popular beach in California, but the number of injuries before and after implementation of spine clipping were not significantly different (Lowe et al. 2007). Infections from stingray barb injuries is a concern, but the microbiomes of stingray barbs do not contain a high abundance of human pathogens (Gonçalves e Silva et al. 2020). Recently, investigation of shark teeth microbiomes revealed species specific microbiomes. Authors suggest that further investigation could allow identification of shark species based on bacteria present in the wound and improve targeted antibiotic treatment (Storo et al. 2021). Despite injuries from stingrays being common, only one study has focused on bacterial communities from stingrays (Gonçalves e Silva et al. 2020). Understanding stinger microbial communities could lead to more targeted antibiotic treatment for stings.

Gill microbiomes are important, especially for elasmobranchs. Elasmobranch gill tissues are more susceptible to environmental toxins than teleost fish (de Boeck et al. 2001, Grosell et al. 2003, Wosnick et al. 2021). Microbes associated with thresher sharks have a higher relative abundance of genes associated with heavy metal resistance when compared

to the water column which may help sharks metabolize toxic compounds (Doane et al., 2017). In *Urobatis jamaicensis* gill microbiomes, Bacteroidetes are abundant in wild rays and lower in abundance in aquarium housed rays (Pinnell et al., 2021). Similarly in wild *Aetobatus narinari*, gill microbiomes have lower diversity in captivity than in the wild (Clavere-Graciette et al., 2022). No investigations of shark or ray gill microbiomes have used shotgun metagenomics, therefore the functions of gill microbes remain unknown. Shotgun metagenomic investigation of gill associated microbial communities would reveal the role of gill microbes and offer insights on the role microbes play in offloading toxins.

Cloaca and gut microbiomes play a large role in animal health and digestion. In sharks, cloaca microbial communities typically have low diversity (Leigh et al. 2021; Pratte et al. 2022; Storo et al., 2021). Low diversity is driven by *Photobacterium damselae* which is highly abundant in cloaca and faecal samples (Pratte et al. 2022; Leigh et al. 2021). Though typically a fish pathogen *P. damselae* appears to play an important role in cloaca microbial communities. Metagenomic analysis showed similar functional gene potential of *C. charcharis* and *R. typus* faecal microbiomes (Pratte et al. 2022). In rays, cloaca microbiomes are less affected by captivity than other body sites, though this is limited to microbial taxa due to 16S sequencing (Clavere-Graciette et al., 2022, Pinnell et al. 2021).

Microbiome trends have been described in primarily in pelagic sharks which possess morphological features called dermal denticles. Dermal denticles are the small toothlike scales that cover the epidermal surface of sharks (Meyer & Seegers 2012). Rays and skates, although closely related to sharks, have skin covered by mucus with sparse or no denticles (Meyer & Seegers 2012) a trait more similar to teleost fish. Microbiomes of rays and skates have received much less attention than their pelagic counterparts. All studies have included

captive rays, with limited information on wild ray microbiomes. Emerging trends suggest lower diversity in captivity and a significantly different community in captive compared to wild individuals (Gonçalves e Silva et al. 2020; Pinnell et al., 2021). Sharks maintain microbial communities similar across captive and wild environments (Goodman et al., 2022) which suggests ray microbiomes are more influenced by environmental variables than sharks. Unlike other Elasmobranchs which spend most or all their life in the water column, rays spend much of their life living on or buried in sediment (O'Shea et al. 2012, Crook et al. 2022). Microbes from both the water column and sediment are in direct contact with rays throughout their life and could affect their microbiome. While the taxonomic composition of Gymnura altavela microbiomes differed from the environment, they were more similar to sediment than water column. G. altavela microbiomes and sediment exclusively shared 100 OTUs while rays and water only exclusively shared 42 OTUs (Gonçalves e Silva et al. 2020). Cownose ray microbiomes were significantly distinct from their environment, including water and sediment (Kearns et al., 2017). While limited, current studies show that while ray microbiomes are distinct from the environment, sediment could play an important role in microbiome composition (Gonçalves e Silva et al., 2020).

Rays are ambassador species in aquariums across the globe. Rays safely interact with the public in touch-tank exhibits and are important organisms for public education (Kearns et al., 2017, Lawrence et al., 2021). Recently, microbiomes have been recognized as an important consideration for conservation biology. Preserving "natural" microbiomes in captivity and understanding the effects of anti/probiotics on captive host microbiomes has implications for conservation (Trevelline et al., 2019). Microbiomes of several vertebrates, including mammals, amphibians, reptiles, and elasmobranchs have been studied in captivety

(Alfano et al., 2015; Chiarello et al. 2017; Chong et al. 2019; Gonçalves e Silva et al. 2020; Goodman et al. 2021; Kearns et al. 2017; Pinnell et al. 2021). Understanding the mechanisms behind changes in microbiomes is important for maintaining host health in captivity (Perry et al., 2021). Many of the ray microbiome studies thus far have occurred in captivity (Gonçalves e Silva et al., 2020; Kearns et al., 2017; Pinnell et al., 2021). These studies provide important information about how the captive environment affects the microbiome of several species of ray. In exhibits where humans physically interact with rays, transfer of microbes becomes a concern. Less than 1.5% of taxa found in cownose ray microbiomes were known to be associated with human skin (Kearns et al., 2017). These results suggest human interaction does not notably introduce foreign microbes, but comparison to rays without interactions with humans is required to confirm this finding (Kearns et al., 2017). Skin microbiomes from wild and captive *Gymnura altavela* individuals were significantly different and captive *G. altavela* had lower bacterial diversity than their wild counterparts (Gonçalves e Silva et al., 2020). *Urobatis jamaicensis* microbiomes from the gills, skin, and cloacae were also significantly more diverse in wild rays, than their captive counterparts (Pinnell et al., 2021). While few studies exist comparing wild and captive elasmobranch microbiomes, emerging trends in rays differ from those of captive sharks. *Triakis semifasciata* (leopard shark) microbiomes did not show significant differences in alpha diversity between captive, semi-captive, and wild sharks. *Triakis semifasciata* microbiomes across environments did not have significantly different relative abundances of taxa or functional genes within their skin microbiomes (Goodman et al. 2021).

To date, several ray microbiomes have been analyzed using 16S rRNA sequencing methods, which have allowed for taxonomic analysis exclusively (Caballero et al. 2020; Gonçalves e Silva et al. 2020; Kearns et al. 2017; Pinnell et al. 2021). Several taxa are hypothesized to be important because of their ability to produce antimicrobial compounds, form biofilms, degrade organic compounds, or aid in osmoregulation (Gonçalves e Silva et al. 2020; Kearns et al. 2017; Pinnell et al. 2021). There is no data about the functional potential of these microbes in association with batoid hosts. Functional gene analysis is necessary to identify specific gene pathways present in the genomes of these potentially important microbes, and whether microbes associated with ray mucus have a higher relative abundance of specific genes compared to microbes in the surrounding environments. In thresher shark (*Alopias vulpinus*) microbiomes, functional gene potential showed a higher relative proportion of genes associated with heavy metal resistance potentially due to biomagnification of heavy metals (Doane et al. 2017). Leopard shark (*Triakis semifasciata*) microbiomes had different taxonomic compositions across several years. Despite changes in taxonomic composition, microbiomes maintained the same functional gene capacity over time (Doane et al 2021). Several studies have explored microbiomes of sharks using shotgun metagenomics (Doane et al. 2017, 2020, 2021; Goodman et al. 2021). Shotgun metagenomics allows for taxonomic and functional gene analysis. While functional genes present in microbiomes are not necessarily expressed, there is correlation between genes represented in metagenomes and those being expressed in metatranscriptomes (Gilbert et al., 2010). The functional potential of *Urobatis halleri* skin microbiome is the only published stingray functional profile available (Doane et al. 2020).

Elasmobranch microbiomes have been reviewed by Perry et al., in 2021, which highlighted several important knowledge gaps. Only 42 out of 1300 species have been investigated previously, and most studies utilized 16S rRNA sequencing, limiting analysis to taxonomy (Perry et al., 2021). The unique evolutionary and morphological features of Elasmobranchs provide an opportunity to close knowledge gaps in this ancient lineage of vertebrates. Because of the publication of a literature review at the start of my thesis (Perry et al., 2021), I have focused my review of the literature associated with the resistance genes including both antimicrobial and heavy metal resistance genes on marine organisms.

Literature review: Antimicrobial Resistance in Marine Host microbiomes

Abstract

Antimicrobial resistance (AMR) is a global environmental and public health concern. Bacteria that survive exposure to antibiotics, disinfectants, or other antimicrobial compounds are deemed “resistant.” These microbes cause infections in humans or other organisms which are difficult or impossible to treat with traditional antibiotics. The introduction and spread of antimicrobial resistance genes (AMGs) in the marine environment impact marine life. This review will discuss how AMR is introduced to the environment and the impacts of on wild and captive marine hosts.

Introduction

Antimicrobial resistance (AMR) is a growing public health and environmental concern. The World Health Organization considers AMR among the top ten public health

threats globally. In 2019 it is estimated that over 1.2 million people died due to infections caused by resistant bacteria (Murray et al., 2022). Antimicrobial resistance causes huge economic (up to 3.8% of global GDP in the next 25 years) and ecological losses due to disease outbreaks (Ahmad et al., 2019). While antibiotic resistance genes arise naturally in the environment, their rapid emergence and prevalence due to the misuse of antibiotics is remarkable (Larsson & Flach, 2022).

Antimicrobials (including antibiotics) are dispersed throughout marine systems via wastewater, untreated sewage, agricultural runoff, and other anthropogenic activity (Berglund, 2015; Lin et al., 2022). Antimicrobials are typically used to treat infections, but in agriculture and aquaculture are used to prevent infection and encourage animal growth (Ibrahim et al., 2019). Eight main groups of antibiotics are approved for clinical use and are commonly used in food production, including aminoglycosides, beta-lactams, glycopeptides, lipopeptides, macrolides, oxazolidinones, quinolones, and tetracyclines (Bush, 2012; Ibrahim et al., 2019). Misuse of antibiotics is increasing in clinical and environmental settings. Antimicrobial use in food production is estimated to increase 67% by 2030, and with an increase in antibiotic use, an increase in AMR is predicted (Ibrahim et al., 2019). Efforts to quantify AMR are growing in response to elevated antimicrobials in the environment (Huijbers et al., 2019; Light et al., 2022). Measuring and managing resistance is challenging because of the diversity of antimicrobial resistant genes (AMG) and microbes, and under reporting of infections caused by resistant bacteria (Hay et al., 2018; Wernli et al., 2017).

Pathways of AMR into the environment

Resistance to antimicrobials (including antibiotics and heavy metal resistance) occurs in the marine environment naturally (Larsson & Flach, 2022). Many host-associated microbes inhibit bacterial growth, to outcompete other microbes within the microbiome and in response to host immune activities (Anteneh et al., 2021; Hentschel et al., 2006). Most hosts produce antimicrobials, which act locally and widespread resistance is not concerning (Larsson & Flach, 2022). AMR and heavy metal resistance become a concern when there is a high influx into the environment via agriculture, aquaculture, sewage and metal deposits, including mining waste, are being discharged into marine systems (Bourdonnais et al., 2022).

Concentrations of antibiotics and other pollutants are typically low in the environment but increase in areas of high human activity (Anim et al., 2020; Meador et al., 2016). AMR bacteria are commonly introduced to the environment via wastewater including sewage, medical and agricultural runoff (Al-Sarawi et al., 2022; Habibi et al., 2022). *Escherichia coli* isolated close to hospital outflow showed overwhelming resistance (98%) to at least one antibiotic, and more than half were multidrug resistant (Al-Sarawi et al., 2022). Abundance of multidrug efflux transporter genes in several bacterial clades found in the nearshore Black Sea suggests AMGs are transferred within and across genera in nearshore bacterial assemblages (Gabashvili et al., 2022). Bacteria isolated from harbours with industrial and agricultural activity in Northern Ireland had higher rates of antibiotic resistance than non-commercial areas (Moore et al., 2013).

Heavy metal contamination associated with human activities is also a concern. Often genes used for heavy metal resistance and AMR have similar molecular mechanisms and are located near each other in the genome or on plasmids (Baker-Austin et al., 2006). Bacteria

isolated from biofilms on experimental metal plates showed an increased in antibiotic and heavy metal resistance as the concentration of copper in the plates increased. (Beleneva et al., 2011). *Vibrio* species cultured from the water column and kelp near industrial areas where copper concentration in the water was higher were more resistant to copper, compared with *Vibrio* species that were cultured from more pristine locations (Busch et al., 2015). The copper exposed microbes were detrimental to the survivorship of kelp recruits (Morris et al., 2016). Synergistic effects of metal and antibiotic-resistant microbes is a concern. Co-occurrence of heavy metal resistance (HMR) and AMR genes on plasmids is most common in communities with long term exposure to antibiotics. Metal concentrations required for co-selection of these genes is unknown, and the role of pollution on stress-induced horizontal gene transfer is also unknown.

Wastewater is an important tool for epidemiology, as drugs including antibiotics, and pathogens like *Vibrio cholera* and most recently Covid-19 can provide information on infection rates and transmission within populations (Choi et al., 2018; Deaver et al., 2021; Okeyo et al., 2018). This also means that wastewater, if not properly treated, is a source of antibiotics and resistant bacteria, including pathogens in the environment (Choi et al., 2018). Meta-anlysis of 17,939 metagenomes collected across the globe revealed that wastewater had the highest abundance of ARGs, but that transmission of ARGs between distinct biomes was low, suggesting there are boundaries preventing the mass spread of ARGs across environments with different characteristics (Lin et al., 2022).

Plastics are routinely introduced to the marine environment and can provide a unique habitat for microbes. Resistant bacteria, including human pathogens, accumulate on the surface of marine plastics and the presence of virulence genes in these microbes suggests

plastics could be a vector for the spread of AMR in the marine environment (Radisic et al., 2020). Plastics are widely dispersed across oceans and potentially spread AMR bacteria across otherwise untraversable routes (Moore et al., 2020). AMR bacteria growing on plastics are present across the globe, even in remote locations such as the Antarctic environment suggesting plastic could be disseminating resistant bacteria to locations of low human activity (Laganà et al., 2019). Microplastics are ingested by marine organisms and humans via seafood and could be a point of transmission across environments and from environment to host (Laganà et al., 2019; Moore et al., 2020; Radisic et al., 2020). In addition, some plastics absorb antibiotics from the environment, providing opportunity for resistance genes to be selected within the colonizing microbial community (Feng et al., 2020).

Aquaculture has the potential of introducing ARGs into the environment. Water discharged from aquaculture facilities utilizing antibiotics introduces antibiotics to the water column where surrounding bacteria can acquire resistance genes (Shah et al., 2014; Tendencia & de La Penã, 2001). Bacterial isolates from shrimp ponds that were currently exposed to antibiotics had the highest rates of resistance and lowest rates of sensitivity when compared to bacteria isolated from ponds that used antibiotics previously or had never been exposed to antibiotics. Many of the resistant species from aquaculture belong to *Aeromonas* and *Vibrio* genera and both clades containing known marine pathogens (Tendencia & de La Penã, 2001). *Vibrio* species isolated form aquaculture regions had a Multiple Antibiotic Resistance (MAR) index greater than 0.2 indicating high levels of resistance and exposure to antibiotics (Mohamad et al., 2019). High MAR index values (>0.2) suggest an organism has been exposed to heavily contaminated environments (Krumperman, 1983; Rose et al., 2009).

Bacteria isolated from farmed organisms including shrimp and shellfish, had a higher proportion of mobile genetic elements than wild teleost fish (Delannoy et al., 2022). Aquaculture facilities using antibiotics even in small amounts affect the presence of ARGs in bacteria. For example, at a Malaysian aquaculture farm, there was a significant correlation between concentration of tetracycline use and tetracycline resistance, which was evident even where the use was at low concentration. In addition, several bacterial strains that were not previously reported to contain tetracycline resistance harboured tet genes, suggesting aquaculture farms utilizing antibiotics contributes to the transmission and evolution of ARGs (Thiang et al., 2022). Prophylactic use of antibiotics facilitates increased resistance in bacteria supported by an increased relative abundance of resistance genes (Patil et al., 2020). Bacterial abundance and resistance were greater near salmon aquaculture facilities, and resistance to antibiotics persisted for up to 1 km from the site using heavy doses of antimicrobials (Buschmann et al., 2012). These results suggest that the area affected by antibiotics and ARGs increases as frequency and dose of antibiotic use increases. Aquaculture has surpassed traditional fishing production, which has increased food availability while reducing reliance on wild fisheries. As aquaculture continues to grow, it may have negative impacts on marine life via ARG transmission and disease outbreaks (Tacon, 2020).

Persistence of AMR in association with marine hosts

Invertebrate Hosts

Invertebrates including non-fishery species like sponges and coral have bacterial communities that display antimicrobial resistance. Most biofilm-producing bacteria associated with 11 species of sponges were resistant to the antibiotics tested. Biofilms have greater resistance to antibiotics because of increased Horizontal Gene Transfer (HGT) and diffusion of toxic compounds (Santos-Gandelman et al., 2013). The human pathogen, *Serratia marcescens*, also infects elkhorn coral, causing devastating loss of coral cover in the Caribbean. *Serratia marcescens* strain PDR60 was isolated from corals and comparison with wastewater strains confirms the transmission of disease from humans to marine organisms (Patterson et al. 2011). Freshwater mussels concentrate bacteria during filtering and have a higher proportion of resistant bacteria than the surrounding water (Saavedra et al. 2022). *Vibrio alginolyticus,* which causes disease in humans and invertebrates, was isolation from cooked prawns was revealed to contain novel resistance genes, which is cause for concern regarding food borne illness and transmission of ARGs in shellfish populations (Morris et al., 2023). *Vibrio* spp. isolated from coral and lobster eggs has a relatively high resistance profile and some strains are pathogenic to humans or marine organisms (Kumari et al., 2020).

Vertebrate Hosts

Fish

Several *Vibrio* species are known fish pathogens, and strains isolated from diseased fish in aquaculture facility showed a multidrug resistance rate of 64% which is greater than expected and is likely to increase over time (Deng et al., 2020). Widely distributed virulence genes present in *Vibrio* spp. isolated from aquaculture indicates these genes can be

transferred between clades (Dang et al., 2009; Mohamad et al., 2019). *Vibrio splendidus* genomes isolated from fish included ICEVspPor3 an integrating conjunctive element that carries tetracycline and mercury resistance genes that can be transferred to other Gammaproteobacteria species (Balado et al., 2013). Plasmids could be contributing significantly to the spread of AMGs in aquaculture facilities (Nonaka et al., 2014), because many plasmids contain multiple resistance genes and transfer readily between microbial species. Plasmid-mediated quinolone resistance genes were found in and near salmon aquaculture sites (Buschmann et al., 2012). Tet(M) resistance is readily spread via pAQU plasmid to *E. coli* (Nonaka et al., 2014). *Vibrio* spp. isolated from Norway water, herring, and bivalves were susceptible to tetracycline and doxycycline, but AMGs for these antibiotics were present in the genomes but those genes were not expressed (Håkonsholm et al., 2020).

Shark microbiomes have higher relative abundance of both heavy metal and antibiotic resistance genes than the surrounding water (Doane et al., 2017, 2022; Goodman et al., 2022). Captive leopard sharks had a significantly higher relative abundance of the resistance gene BlaR1 than semi-captive and wild populations, suggesting exposure to humans is contributing to more resistance within the microbiome of captive fish (Goodman et al., 2022). Co-selection of ARG and heavy metal resistant genes has been observed in the environment and microbes associated with other fish species but has not been tested in sharks (Balado et al., 2013; Seiler & Berendonk, 2012). A *Pseudomonas* species isolated from thresher shark was resistant to 8 antibiotics. External tissues generally (skin) had bacteria with greater resistance while internal tissues had a higher proportion of isolates with

no resistance to antibiotics (Rose et al., 2009). This suggests the surrounding environments could be contributing to resistance in external microbiomes, potentially increasing the risk of infection for marine hosts.

Birds and Reptiles

Marine birds, specifically gulls, have populations that interact closely with humans. AMR passed between gulls and humans is a public health and environmental concern. Metagenomics detected a large proportion of previously undetected AMGs in gull faeces. Presence of AMGs commonly found in human bacteria recovered from gull faeces suggests an impact of human activity on natural AMR in wild gulls (Martiny et al., 2011). A large proportion of kelp gull (*Larus dominicanus*) faecal samples showed resistance to multiple antibiotics while fewer penguin microbiomes showed multiple resistance. (Ewbank et al 2021). The class 1 integron contributes to the dissemination of AMR in the environment. This integron was significantly more prevalent in captive little penguins (44.7%) than wild little penguins (3.2%) suggesting that exposure to humans, or anthropogenically impacted water sources are introducing AMR to these populations (Lundback et al 2021).

Sea turtles are considered an indicator species because their feeding, mating, and lifespan are heavily impacted by pollution and fishing. Their affinity for coastal habitats and their relationship with ecosystem health lends them well to AMR monitoring (Alduina et al., 2020; Blasi et al., 2020; Foti et al., 2009). All gram-negative isolates from cloaca swabs of loggerhead sea turtles (*Caretta caretta*) were resistant to at least one antibiotic, with *Pseudomonas* isolates having the greatest rates of resistance (Foti et al., 2009). No difference in antibiotic resistance was detected between isolates from healthy and weak *C.*

caretta individuals (Blasi et al., 2020). Metagenomic analysis of *C. caretta* and the surrounding environment found blaTEM, the gene for beta-lactam resistance, in 82% of samples, but tetA and qnrS, the genes for tetracycline and quinoline resistance, were only detected in live turtle samples (Alduina et al., 2020). The abundance of AMR in turtle microbiomes is concerning due to their migratory behaviour and proximity to humans which could lead to the spread of AMGs (Alduina et al., 2020). Galapagos iguanas had low abundance, but high diversity of ARGs. Presence of resistant pathogens in the gut microbiome of iguanas is still a concern, as the risk of AMR spreading to pristine environments increases (Vasco et al 2022).

Mammals

AMR in marine mammals poses a risk to wild and captive populations and transmission from animals to humans during interactions is increased compared to other organisms. Eight *E. coli* isolates from southern resident killer whale (*Orcinuc orca*) faeces were resistant to tetracycline, three of which could transfer *tet*B to recipient *E. coli* (Melendez et al., 2019). Chinook salmon, orcas main prey source, are exposed to antibiotics as juveniles due to their residence in estuaries that receive outflow from waste-water treatment plants (Meador et al., 2016). Presence of potentially pathogenic and resistant *E. coli* in the gut suggests that whales may be exposed via wastewater discharged to the environment or consumption of prey (Chinook salmon) directly (Meador et al., 2016; Melendez et al., 2019). A case study on beached short, finned pilot whales (*Globicephala macrorhynchus*) found whales and human volunteers shared a highly similar *Staphylococcus aureus* strain (USA300). This strain is known to be spread from humans to animals and vice

versa, but the origin of MRSA (methicillin-resistant S*taphylococcus aureus*) in this instance could not be resolved (Hower et al., 2013). Bottlenose dolphins (*Tursiops truncates*) living in more polluted areas had higher antibiotic resistance than unimpacted areas, and greater rates of antibiotic resistance than the surrounding water (Shen et al 2020). Multidrug resistant *Morganella morganii* poses a threat to wild and captive dolphin populations and could be potentially transmitted to humans (Park et al., 2020). This is consistent with trends where AMR and the occurrence of potential pathogens is higher near areas of human activity than more remote locations (Jurelevicius et al., 2021; P. Moore et al., 2013).

A case study on a lethal infection of a common seal pup (*Phoca vitulina*) showed high similarity of a *Klebsiella pneumoniae* plasmid isolated from the seal pup and the *K. pneumoniae* plasmid from human bacteria. This suggests wastewaters are contaminating the environment and affecting marine life, even where there are small numbers of infections (Duff et al., 2020). Sea lions (*Neophoca cinera*) living at remote sites in South Australia have *E. coli* phylotypes found in humans, one of which is commonly associated with ExPEC disease. Pups with hookworm are more susceptible to ExPEC suggesting that sea lions exposed to anthropogenic activity are more susceptible to disease (Fulham et al., 2018, 2022). Seals are also potential vectors for AMR, as several species of seal carried pathogenic *E. coli* (Gross et al., 2022).

Humans, while not marine mammals, are still affected by AMR in the marine environment. Specific pathogens, have existing resistance genes and are characterized by their ability to infect host organisms and quickly acquire virulence genes including resistance (Dubey et al., 2022; Kim et al., 2022; Wu et al., 2008). Some pathogens like those from the *Vibrio* genus have been historically involved in deadly infections in marine

organisms and humans (Frans et al., 2011; Okeyo et al., 2018). Tet(M) the gene responsible for tetracycline resistance can be transferred from marine bacteria to human pathogens including *E. coli* and *E. faecalis* (Neela et al., 2009). Densities of resistant *E. coli* are typically higher in enclosed bays and near estuaries, areas humas tend to frequent (da Costa Andrade et al., 2015). Humans, specifically surfers, are at higher risk of acquiring potentially pathogenic and resistant *E. coli* from seawater than non-surfers. While this risk is lower than transmission in hospital settings, as AMR becomes more prevalent in the environment, this risk becomes more important (Leonard et al., 2018). Potential human pathogens were detected in the environment but there was a low frequency of resistance phenotypically, but bacteria had resistance genes in their genomes and could be acting as reservoirs for AMGs in the environment (Håkonsholm et al., 2020).

Global Monitoring and Mitigation

Introduction and persistence of AMGs in global oceans has consequences for human and environmental health. Identifying critical environments, like tropical gulfs, for AMR emergence and dissemination will help guide mitigation (le Quesne et al., 2012). Levels of antibiotic resistance in common bacterial pathogens infecting marine mammals increased between 2004 and 2010. *E. coli* had a significant increase in AMR to half of the antimicrobial classes tested over time (Wallace et al., 2013). Identifying and monitoring reservoirs of resistance, including sentinel hosts, is an important aspect of spread and mitigation. Culturing techniques are useful but are time intensive. Culture independent techniques are becoming more accessible and affordable. For example, quantitative PCR (qPCR) can be used to screen a sample for specific known genes (Abramova et al., 2023).

Global metagenomics monitoring will contribute to managing AMR and can be complemented with transcriptomics to track and mitigate future spread of AMGs (Danko et al., 2021). Metagenomics can be useful for the discovery and description of AMGs, but without phenotypic testing, it is unknown if the genes are being expressed (Martiny et al., 2011; Yu et al., 2022). For example, *Vibrio* genomes from Norway contained AMGs but when cultured were susceptible to antibiotics (Håkonsholm et al., 2020). Designing a global monitoring procedure is difficult and expensive, but simplified and standardized monitoring can guide public and environmental health efforts (Light et al. 2022). Combining metagenomics and tracking survey of wild marine animals, such as sharks or turtles, may provide a tool to track the susceptibility and potential spread of antimicrobial resistant genes in the oceans. Monitoring and mitigation efforts are essential for reducing ecological, economic, and health impacts of widespread antimicrobial resistance.

Thesis Aims

Using the review of Elasmobranch microbiomes by Perry et al., 2021 and my review of the AMR literature, I have addressed knowledge gaps in Elasmobranch microbiome research over 6 chapters. I first describe taxonomic and functional potential of skin, gill, and cloaca microbial communities from a demersal shark and ray (Chapter 1). I investigate the microbiomes of juvenile Elasmobranchs across two sampling dates for four species (Chapter 2). I compare two host's skin microbiomes across two locations (Chapter 3). I identify host mucus compounds which influence the microbial community (Chapter 4). I isolated and sequenced individual bacteria, improving database resolution (Chapter 5). Finally, I compare skin microbiomes of global populations of benthic Elasmobranchs using single-

read and metagenomic assembly and explore the extent of antimicrobial and heavy metal resistance genes across a broad range of species (Chapter 6).

CHAPTER 1: SLIMY VS SCALY: IDENTIFING MICROBIOME DIVERSITY AND FUNCTIONS ACROSS SKIN, GILL AND CLOACA IN TWO BENTHIC ELASMOBRANCHS

Context

Host microbiomes are influenced by host specific and environmental factors, but internal and external body-sites are affected differently. Elasmobranch skin, gill, and cloaca microbial communities are generally distinct from each other taxonomically, but there have been no previous investigations on microbiome functions, except in the cloaca of pelagic sharks. To address this knowledge gap, I describe the taxonomic and functional potential of skin, gill, and cloaca microbial communities using shotgun metagenomics. I compare two benthic hosts, *Myliobatis tenuicaudatus* and *Heterodontus portusjacksoni*. This is the first investigation of benthic Elasmobranch body site microbial functions and the first time novel Elasmobranch prophages have been identified in *Photobacterium damselae*. This work is being prepared for submission to Animal Microbiome.

Abstract

Host microbiomes are influenced by both host metabolism (e.g., physiology or diet) and environmental (e.g., temperature or pH) factors. To understand the impact of host and environmental factors on microbiome characteristics, I compared microbiomes across three distinct body sites in two co-occurring species of Elasmobranchs. The host species, *Myliobatis tenuicaudatus* (southern eagle ray) and *Heterodontus portusjacksoni* (Port Jackson shark), which share the same rocky reef habitat and similar diets in southern Australia. The host have variable epidermal morphology, mucus production and life history.

I used shot-gun metagenomics to compare microbiomes of the highly environmentally exposed epidermis, and two less exposed tissues gills and cloaca of both host species to explore the level of influence of host verses environment. *Myliobatis tenuicaudatus* gills had distinct microbial families and functional composition to skin and cloaca. *Heterodontus portusjacksoni* had distinct microbial family communities but similar functional profiles across body sites. When comparing hosts to each other, skin, gill, and cloaca microbial family communities were significantly different. Gill and cloaca microbial functions were different between hosts, but skin functional potential was similar. Five Metagenome Assembled Genomes (MAGs) were recovered from *M. tenuicaudatus* and belonged to *Vibrionaceae* and *Alteromonadacae* clades, while one *Psychrobacter* MAG was recovered from *H. portusjacksoni*. Host internal body sites (gill and cloaca) were most distinct between host species in both taxonomy and functional potential suggesting they are more influenced by host factors than the environment. Skin microbial functions remined similar across hosts suggesting those communities are generalists and more influenced by the environment.

Introduction

Microbiomes are the community of bacteria, archaea, and viruses that live on or within a host's tissues (Berg et al., 2020). The "holobiont" considers a host and associated microbes as one entity (Rohwer et al., 2003). Host microbiomes are specific for each body-site or tissue, e.g., skin, gut, lungs, gills, where the microbes provide specific functions (Kennedy & Chang 2020). Tissue specific microbiomes have been well studied in humans (especially in the gut) due to the important and complex role microbiomes play in health and disease (Ha et al., 2014). In marine systems, microbiomes are dynamic communities

influenced by host specific factors like genotype (Bonder et al., 2016) and evolutionary history (Brooks et al., 2016) but are also impacted by environmental factors like temperature (Lima et al., 2020) and habitat (Kim et al., 2021).

Microbiomes of co-occurring host species are influenced by environmental and host variables to varying degrees. In wild rodent microbiomes, host species had a stronger effect on gut microbiome variability than field site, although both significantly influenced microbiomes (Anders et al., 2021). Six Madagascar mammal hosts had significantly different gut microbiomes, but habitat use and diet played important roles in these distinctions. Hosts with the most distinct diets had more dissimilar microbiomes, however, in shared habitats host microbiome dissimilarity was reduced, indicating that both host and environmental influence on gut communities (Perofsky et al., 2018).

In marine hosts, such as dolphins, the oral microbiomes were more similar between wild and captive populations than between dolphins and sea lions from the same enclosure. Therefore, host drives differences between species but within a species the environment plays a significant role in shaping microbial communities (Bik et al 2016). *Jaera*, a clade of intertidal isopods, showed significant microbiome differences across regions hypothesised to be the result of changes in abiotic conditions (Wenzel et al., 2018). Therefore, across clades hosts are the main drivers of microbiomes, but within a clade or species, the environment plays significant roles in shaping host microbial communities.

These broad eco-evolutionary patterns of host-microbiome relationships are complicated when considering the microbiome of different body-sites. Microbial communities within a species often vary across host body sites such as skin, digestive tract, and respiratory tract (Bik et al., 2016). In corals, surface mucus, tissues, and skeleton have

different core microbiomes which were influenced primarily by host but were impacted differently by environmental variables (Pollock et al., 2018). Distinguishing morphological traits may influence the microbial communities across different body sites, for example, denticle morphology is hypothesized to affect shark and ray epidermal microbial communities (Doane et al., 2017; Kerr et al., 2023; Mika at al., 2021). Within the digestive tract of *Sphyrna tiburo* (bonnethead shark), microbiomes of gut regions were significantly different, but microbial communities did not differ between wild and captive sharks (Leigh et al., 2021). The interplay between host and environment will be complicated by body site and host species investigated.

In the marine environment, an organism's external body sites, like skin, are continuously contending with invasion of microbes suspended in the water column (Dash et al., 2018). Elasmobranchs typically have skin microbial communities that are species specific and distinct from their environment (Doane et al., 2017; Gonçalves e Silva et al., 2020; Kerr et al., 2023). In wild *Triakis semifasciata* (leopard sharks), bacterial taxa on the skin fluctuated over three sampling events, but functions remained stable (Doane et al., 2022). The taxonomy of epidermal microbial communities of *Rhincodon typus* (whale shark) varied across aggregation site, but underlying community structure remained consistent (Doane et al., 2023). *Urobatis halleri* (bat ray) and *Carcharhinus melanopterus (*black tip reef shark) epidermal microbial taxonomy varied across sampling locations, and in *U. halleri* functions remained stable (Kerr et al., 2023; Pogoreutz et al., 2019). Despite location specific differences, *C. melanopterus* healthy and injured skin microbiomes were similar, indicating host and environment is influencing epidermal microbiomes simultaneously (Pogoreutz et al., 2019). *Aetobatus narinari* (spotted eagle ray) alpha and beta diversity in

epidermal microbiomes were significantly lower in aquarium compared to wild rays, but *T. semifasciata* maintained epidermal microbiome diversity in aquaria (Clavere-Graciette et al., 2022; Goodman et al., 2022) indicating shark and ray skin communities are affected by the environment in different ways. Epidermal microbial communities also have different functional capacity than the water column. Southern California Elasmobranchs, *T. semifasciata*, *Alopias vulpinus* (thresher sharks), *U. halleri* and *Myliobatis californica* (bat ray), have an increased proportion of heavy metal resistance genes compared to the water column, hypothesized to help the host adapt to polluted environments (Doane et al., 2017, 2022; Goodman et al., 2022; Kerr et al., 2023). Therefore, the environmental impacts on the skin microbiomes varies between sharks and rays, potentially because of skin structure and chemical composition of host mucus.

Gills are also an external organ continuously responding to stress from acute and chronic changes in environmental conditions (Rosa et al., 2017). Water passes over the gills allowing for gas exchange and excretion of waste (Wegner 2015). Elasmobranch gills are dominated by Proteobacteria (now termed Pseudomonadota) (Pinnell et al., 2021; Storo et al., 2021). The genera *Pseudoxanthomonas* occurs on the skin and gills of *Carcharhinus leucas* (bull sharks) and had genes required to degrade harmful organic compounds and heavy metals supporting shark health (Black et al., 2021). Bonefish gill microbiomes are influenced by geographic location and anthropogenic pressures (Campbell et al., 2023). In fish, bacterial taxa involved in nitrogen metabolism (ammonia oxidation and denitrification) are present in the gill microbiome, potentially reducing ammonia concentrations (van Kessel et al., 2016). Reef fish gill microbiomes were driven by the host individual and life stage (Pratte et al., 2018).

Microbiome in internal body sites, like the gut, are influenced by different factors than external microbiomes on the skin and gills. In fish, gut microbial communities are more influenced by diet compared with gill microbial communities (Pratte et al., 2018). Cloaca microbiomes of wild and captive rays are less diverse than skin and gill microbiomes but remained stable between captive and wild populations (Clavere-Graciette et al., 2022). Fish gut microbiomes are distinct from external skin and gill microbiomes (Lowrey et al., 2015; Sylvain et al., 2020). Shark and ray gastrointestinal tract microbiomes are dominated by Vibrio, specifically *Photobacterium damselae* (Clavere-Graciette et al., 2022; Leigh et al 2021; Pratte et al., 2022). Microbial communities in *Carcharodon carcharias* (white shark), and *R. typus* faeces are similar despite differences in diet, suggesting a conserved mechanism for assembling gut microbial communities in sharks (Pratte et al., 2022). *Photobacterium damselae* are present in high abundance in gut microbiomes of at least five Elasmobranch species (Clavere-Graciette et al., 2022; Leigh et al 2021; Pinnell et al., 2021; Pratte et al., 2022). Similarity in gut microbiomes across clades suggests that factors shaping the taxonomic composition of shark gut microbiomes is driven by shark-specific host factors like phylogeny or gut anatomy rather than diet.

Heterodontus portusjacksoni (Port Jackson shark) and *Myliobatis tenuicaudatus* (southern eagle ray) are both benthic Elasmobranchs with overlapping distributions in southern Australia. *Heterodontus portusjacksoni,* are small benthic sharks reaching up to 1.5 meters (Last & Stevens 1994). *Myliobatis tenuicaudatus* are large dorsoventrally flattened rays; mature individuals having disc widths over one meter (Marcotte 2014). Both species have diets dominated by benthic invertebrates, influenced by location and size (age) (Sommerville et al., 2011). Rays create feeding pits where they use their gills and spiracles

to jet water uncovering prey items like crustaceans, polychaetes, and molluscs buried in sediment (Gregory et al., 1979; Sommerville et al., 2011). *Myliobatis tenuicaudatus* at the sampling site in this study are known to scavenge from fishers, especially squid (Roberts personal observation). The diet of both hosts shifts with increased size, larger *H. portusjacksoni* consume more gastropods and echinoderms as bite force increases in larger individuals, while larger *M. tenuicaudatus* individuals consumed larger cephalopods and teleosts (Sommerville et al., 2011).

Although both hosts have similar ecological niches, they have unique life history and morphological characteristics. An important distinguishing characteristic of Elasmobranchs are their dermal denticles. Denticle shape, size and density vary across parts of the body like head, fins, and tail, while some species lack them completely (Ankhelyi et al., 2018; Meyer & Seegers 2012). Microbiome characteristics vary with dermal denticles and mucus content (Doane et al., 2020; Goodman et al., 2024; Kerr et al., 2023). *Heterodontus portusjacksoni* have small crown-shaped denticles (Figure 1.1a), while *Myliobatis tenuicaudatus* have no visible denticles on their dorsal wings (Figure 1.1b). Both species also differ in reproductive strategy. *Heterodontus portusjacksoni* are oviparous, laying eggs in the spring, incubating for 10-11 months before hatching (Rodda & Seymour 2008). *Myliobatis tenuicaudatus* are viviparous (give live birth) (Hartill 1989).

Here I compare the skin, gill, and cloaca microbiomes of *Myliobatis tenuicaudatus* and *Heterodontus portusjacksoni,* to explore the eco-evolutionary role in host microbiome characteristics. I hypothesize that hosts will have comparatively more influence on their microbiomes than the surrounding water column microbiome, and therefore the two species will display significant differences in taxonomic and functional composition of the

microbiomes. We also hypothesize that there will be more variability within *Myliobatis tenuicaudatus* microbiomes than *Heterodontus portusjacksoni* due to rays having a thicker mucus layer. We predict skin and gill microbiomes will be influenced to a greater degree by the environment due to the constant exposure to water column microbes. We predict cloaca microbiomes will be similar due to the evolutionary relationship seen in other shark species, specifically with the bacterium *Photobacterium damselae*. We compare *P. damselae* Metagenomic Assembled Genomes (MAGs) obtained from *M. tenuicaudatus* and other Elasmobranch hosts.

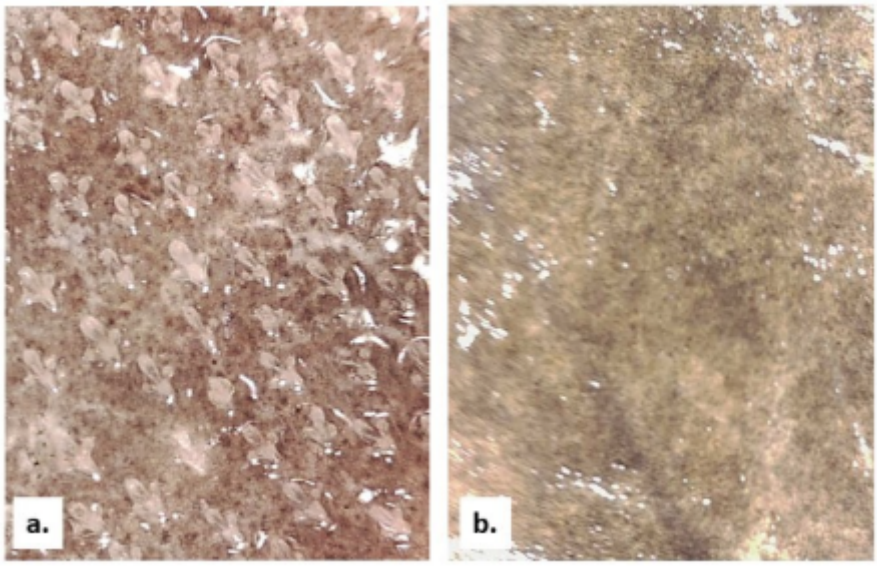

Figure 1.1: Crown shaped dermal denticles on *Heterodontus portusjacksoni* dorsal skin (a.) smooth mucus covered surface on *Myliobatis tenuicaudatus* dorsal skin (b.).

Methods

Sample Collection

Nine female *Myliobatis tenuicaudatus* individuals were caught one-by-one using a seine net at Seacliff Beach, South Australia. Ten female *H. portusjacksoni* individuals were caught by hand at Christies Beach, South Australia, 16 kilometres south of Seacliff Beach. Nylon swabs were gently passed over the desired surface of each organism to collect gill,

cloaca, and skin microbes and stored on ice (Clinton et al., 2021). Two swabs were collected per body site (eg. skin, gills, cloaca) and combined to make one metagenomic sample. Photos were taken of skin of both species using Dino-Lite Premier AF3113T field microscope before animals were released.

Microbial DNA extraction and Sequencing

DNA extraction using the Macherey-Nagel Nucleospin Tissue kit has been modified to accommodate for two swabs per sample. Briefly, 270 µL of T1 buffer and 37.5 µL of Proteinase K are added to a 1.5 mL microfuge tube containing one swab. Samples were incubated overnight at 55 ºC. Then, 300 µL of B3 was added to each tube and incubated at 70 ºC for 10 minutes. Swabs were placed into a spin basket and spin dry for one minute at 14000 rpm, dry swabs and spin baskets were discarded. To each tube 300 µL of 100% ethanol was added and shaken gently. Lysate from both replicate swabs was added to one column 550 µL at a time and spun at 14000 rpm for 1 minute, then flow through was discarded. This step was repeated until total sample volume was loaded on to the filter column. Then, 500 µL of BW buffer was added to each column and spun at 14000 rpm for one minute, then flow through was discarded. Next, 600 µL of B5 buffer was added, spun at 14000 rpm for one minute and flow through was discarded. Columns were spun dry for 2 minutes at 14000 rpm and flow through discarded. Filter columns were transferred to elution tubes where 50 µL of 70ºC BE buffer was added to the filter column and incubated at room temperature for 5-10 minutes. Columns were then spun at 14000 rpm for one minute. This step is repeated to obtain 100 µL of DNA from each sample. Supersucker samples were extracted using the same Macherey-Nagel Nucleospin Tissue kit following Kerr et al., 2023. DNA concentrations were quantified for each sample using Qubit Fluorometer (Invitrogen, USA). Samples were sent to the South Australia Genomics Center as raw DNA where

sample libraries were prepared using the Nextera XT Kit (Illumina, USA) and sequenced on the MGI FCL 2x150 DNBSeq-G400 (MGI, Guangdong).

Bioinformatics

Sequence data was processed using the Atavide pipeline for quality control, annotation and assembly (https://github.com/linsalrob/atavide) (Roach and Edwards 2021). First the pipeline utilizes fastp to quality control sequences, removing sequences with N bases and those less than 150 bp (Chen et al., 2018). Minimap2 (Li 2018) and samtools (Li et al., 2009) were used to filter host sequences using available Elasmobranch reference genomes. Then sequences were annotated using MMseqs (Steinegger & Soding 2017) against the UniRef50 database (Suzek et al., 2007). All bioinformatics was conducted on Flinders University HPC Deepthought (Flinders University). Metagenomes were assembled individually with MegaHit (Li et al., 2015) and binned with VAMB (Nissen et al., 2021). CheckM was used to identify Metagenome Assembled Genomes with at least 70% completeness and less than 10% contamination (Parks et al., 2014). MAGs were annotated using the BVBRC Genome annotation tool (Olson et al., 2022).

To compare *Photobacterium damselae* to other strains found in Elasmobranch MAGs, shotgun sequences from Pratte et al., 2022 were downloaded from the SRA (BioProject PRJNA649531). Following the methods described in Pratte et al., 2022, Megahit was used to co-assemble all samples into contigs (Li et al., 2015). Then, Anvio metagenomics workflow was used annotate and bin contigs using MaxBin2 (Eren et al., 2021; Wu et al., 2016). *Photobacterium damselae* MAGs were uploaded to BVBRC and annotated using the Genome Annotation Tool (Olson et al., 2022). BVBRC comparative systems service was used to compare genes present only Elasmobranch-derived MAGs compared to reference *Photobacterium damselae* strains. Next phold

(https://github.com/gbouras13/phold) was used to annotate prophage genes using default parameters (Bouras et al., 2024). MAG 22969 was vizualized using Proksee (Grant et al., 2023).

Statistical Analysis

Samples with fewer than 2,000 annotated reads or Good's coverage of less than 98% were eliminated from further analysis (Supplementary Table 1.1). Singletons (taxa that have only one read in the dataset) were removed. Taxonomic and functional annotations were standardised on the total number of annotated reads per sample (relative abundance), allowing for comparison across samples with different quantities of reads. Where taxa could not be identified to the family level, the next most specific taxon (e.g. Order) was used and denoted with unknown.

To compare body-site family microbial communities, relative abundances were square root transformed and Bray-Curtis dissimilarity was calculated using Primer7 (version 7.0.23) (Primer-E, UK). Primer7 was used to create a PERMANOVA designed with factor host (two levels: M. tenuicaudatus and H. portusjacksoni) and body-site (three levels: skin, gills, and cloaca) were fixed factors, and individual (18 levels) was nested within host as a random factor.

PERMDISP function was used to compare variability across hosts and body-sites. The pairwiseAdonis package was used to test for body-site differences within host species (Martinez et al., 2020). Bray-Curtis distances were visualized with NMDS plots created with ggplot2 (v. 3.5.1) (Wickham 2016). PAIRWISE adonis was used to compare skin, gills, and cloaca communities within and across host body sites. The DESeq2 package (v. 1.46.0) was used to compare differentially abundant families and functional pathways across host body sites (Love et al., 2014). The mean relative abundance of each family or function within a

body site was calculated and the abundant taxa and functions were plotted as grouped bars (Schloss 2023).

Hill diversity was calculated in R using the vegan package (Wickham et al., 2016). Shapiro-Wilk and Levene's tests showed assumptions were not met for all comparisons therefore differences between host body site Hill diversity within a species was tested using Kruskal Wallis non-parametric tests. Welches t-test was used to compare the same body site across the two hosts.

Results

After filtering and quality control, 45 samples collected from ten *M. tenuicaudatus* and nine *H. portusjacksoni* individuals were used for analysis (Supplementary Table 1.1). Across all samples 38,110,590 reads were annotated with an average of 732,896 reads per sample. The dataset contained 192 unique phyla and 1250 unique families across all microbiomes. Pseudomonadota was the most abundant phylum in all microbiome types. Pseudomonadota had a mean relative abundance of 64.32 %, 53.34 %, and 52.41 % in *M. tenuicaudatus* cloaca, skin, and gills respectively. In *H. portusjacksoni,* Pseudomonadota relative abundance was 41.40 %, 39.10 %, and 30.62 % in gills, cloaca, and skin, and gills respectively. Other abundant phyla include an unknown Bacteria phylum making up an average of 26.52 % of *H. portusjacksoni* microbiomes and 18.38 % of *M. tenuicaudatus* microbiomes. Bacillota, Bacteroidota, and Planctomycetota made a relative contribution of at least 5% of the total microbiome. Abundant bacterial families include *Vibrionaceae*, *Moraxellaceae*, and *Aeromonadaceae* (Figure 1.2).

Body site microbial communities compared between hosts

Overall, the hosts had significantly different microbial families and functional potential (PERMANOVA Pseudo-F = 9.4768, df = 1, denominator df = 22.8, p = 0.001 & PERMANOVA Pseudo-F= 6.4224, df = 1, denominator df = 24.44, p = 0.001 respectively). Body sites showed different microbial families and functional potential (PERMANOVA

Pseudo-F = 2.6365, df = 2, denominator df = 23, p = 0.008, & PERMANOVA Psuedo-F = 4.2514, df = 2, denominator df = 23, p = 0.001 respectively). Interaction terms accounting for individual and between host species and body site were not significant for taxonomic communities (PERMANOVA Pseudo-F = 1.1053, df = 1, denominator df = 23, p = 0.313 & PERMANOVA Pseudo-F = 1.9325, df = 1, denominator df = 23, p = 0.048). For functional communities individual was not significant (PERMANOVA Pseudo-F = 1.151, df = 16, denominator df = 23, p = 0.234) but the interaction term for host and body-site was significant (PERMANOVA Pseudo-F = 2.2965, df = 2, denominator df = 23, p = 0.018).

Both species also had significantly different dispersion in the microbiome (PERMDISP F = 7.28, df = 2, p = 0.003) (Figure 1.4). The alpha diversity (Hill diversity) of the skin and gill microbiomes in *Myliobatis tenuicaudatus* and *H. portusjacksoni* were not different (W = 17, df = 1, p = 0.94 and W = 55, df = 1, p = 0.07 respectively) but cloaca diversity was significantly lower in *M. tenuicaudatus* (W = 48, df = 1, p = 0.02) (Supplementary Figure 1.2).

***Myliobatis tenuicaudatus* microbiomes**

Myliobatis tenuicaudatus had significantly different microbial families across body sites (PERMANOVA F = 2.93, df = 2, p = 0.015) (Figure 1.3a). Pairwise comparisons showed differences between *M. tenuicaudatus* gills and both skin (PERMANOVA F = 3.61, df = 1, p-adj = 0.004) and cloaca (PERMANOVA F = 4.46, df = 1, p-adj = 0.015). The microbial families present in the cloaca were highly variable across individuals and therefore there was no significant difference between skin and cloaca microbial communities measured at the family level (PERMANOVA F = 1.31, df = 1, p-adj = 0.828) (Figure 1.4).The microbiomes of *Myliobatis tenuicaudatus* had similar Hill diversity across body sites (Kruskal-Wallis chi-squared = 1.4219, df = 2, p = 0.49) (Supplementary Figure 1.2).

In the microbiomes of the gills, *Aeromonadaceae*, *Carnobacteriaceae*, and *Morganellaceae* (mean 22.6%, 9.04%, and 6.68% respectively) were highly abundant bacterial families. *Vibrionaceae* and *Aeromonadaceae* (mean 19.5% and 8.08% respectively) were abundant identified families in the cloaca. Skin communities also had high mean relative abundances of *Aeromonadaceae* (6.26%) and *Moraxellaceae* (7.09%). An unknown Gammaproteobacteria clade was abundant in the cloaca (14.48 %), skin (9.92 %) and gills (6.71 %) (Figure 1.5a).

Functional differences were also observed across body sites in *M. tenuicaudatus* (PERMANOVA F = 4.59, df = 2, p-adj = 0.002) (Figure 1.3b). Pairwise comparisons showed gill functional gene potential was significantly different from skin (PERMANOVA F = 4.87, df = 1, p-adj = 0.012) and cloaca (PERMANOVA F = 7.37, df = 1, p-adj = 0.006). There was no significant difference between functional potential of skin and cloaca microbiomes (PERMANOVA F = 1.44, df = 1, p-adj = 0.567).

Myliobatis tenuicaudatus gill microbiomes had a high relative abundance of lysine fermentation (4.21 %) compared to skin (1.53 %) and cloaca (1.25 %) microbiomes. Gill microbiomes also had a high relative abundance of Metabolite repair, Heme O and A biosynthesis, Sporulation Cluster genes compared to skin and cloaca microbiomes (Figure 1.5). Flagellum genes were more abundant in cloaca (1.17 %) and skin (0.88 %) microbiomes compared with in gill microbiomes (0.50 %). Branched chain amino acid biosynthesis had a higher mean relative abundance in the cloaca (1.06 %) and skin (0.96 %) microbiomes compared with the gill microbiomes (0.55 %). Genes associated with cell division had the same mean relative abundance in skin and cloaca microbiomes (1.20 %) and was less abundant in gill microbiomes (0.77 %) (Figure 1.5).

Heterodontus portusjacksoni microbiome

Heterodontus portusjacksoni microbial communities were significantly different across body sites (PERMANOVA F = 2.73, df = 2, p = 0.001) (Figure 1.3a). Pairwise comparisons showed differences between all three body sites: skin vs. gills (PERMANOVA F = 3.24, df = 1, p-adj = 0.009) skin vs. cloaca (PERMANOVA F = 3.46, df = 1, p-adj = 0.003) and cloaca vs. gill (PERMANOVA F = 2.07, df = 1, p-adj = 0.018). The microbiomes from *Heterodontus portusjacksoni* had similar Hill diversity across body sites *(*Kruskal-Wallis chi-squared = 4.722, df = 2, p = 0.09). In the cloaca, *Moraxellaceae* (mean 5.25%) was among the most abundant bacteria that could be identified to the family level. Unidentified Gammaproteobacteria and Pseudomonadota, had similar average relative abundance in cloaca samples (mean 7.18% and 6.81% respectively). *Heterodontus portusjacksoni* gill microbiomes had a high mean relative abundance of unknown bacteria (24.52%), Pseudomonadota unknown (7.17%), and *Moraxellaceae* (6.72%). Unidentified families belonging to Planctomycetota (6.03%), Pseudomonadota (6.00%), and Gammaproteobacteria (5.94%) were among the top five most abundant taxa on the skin (Figure 1.2).

Microbial functional composition was similar across *H. portusjacksoni* body site microbiomes (PERMANOVA F = 1.71, df = 2, p = 0.09). Pairwise comparisons confirmed similarity in functional potential (p-adj > 0.05 for all comparisons). Branched chain amino acid biosynthesis, cell division cluster, putative oxidase COG2907, TCA cycle and universal GTPases all had similar relative abundances across body sites (Figure 1.5). Heme O and A biosynthesis was more abundant in gill microbiomes (11.17 %), compared with the cloaca (7.21 %), and skin (2.57 %) microbiomes. NADH ubiquinone oxidoreductase, multi subunit cation antiporter, and respiratory complex I were all more abundant in gill microbiomes

(3.88 %, 3.94 %, and 3.90 %) than in cloaca (2.57 %, 2.64 %, and 2.61 %) or skin (1.15 %, 1.25 %, and 1.18 %) microbiomes. Skin had the highest relative abundance of gram-negative cluster (3.93 %) followed by gills (2.72 %) and cloaca (2.30 %).

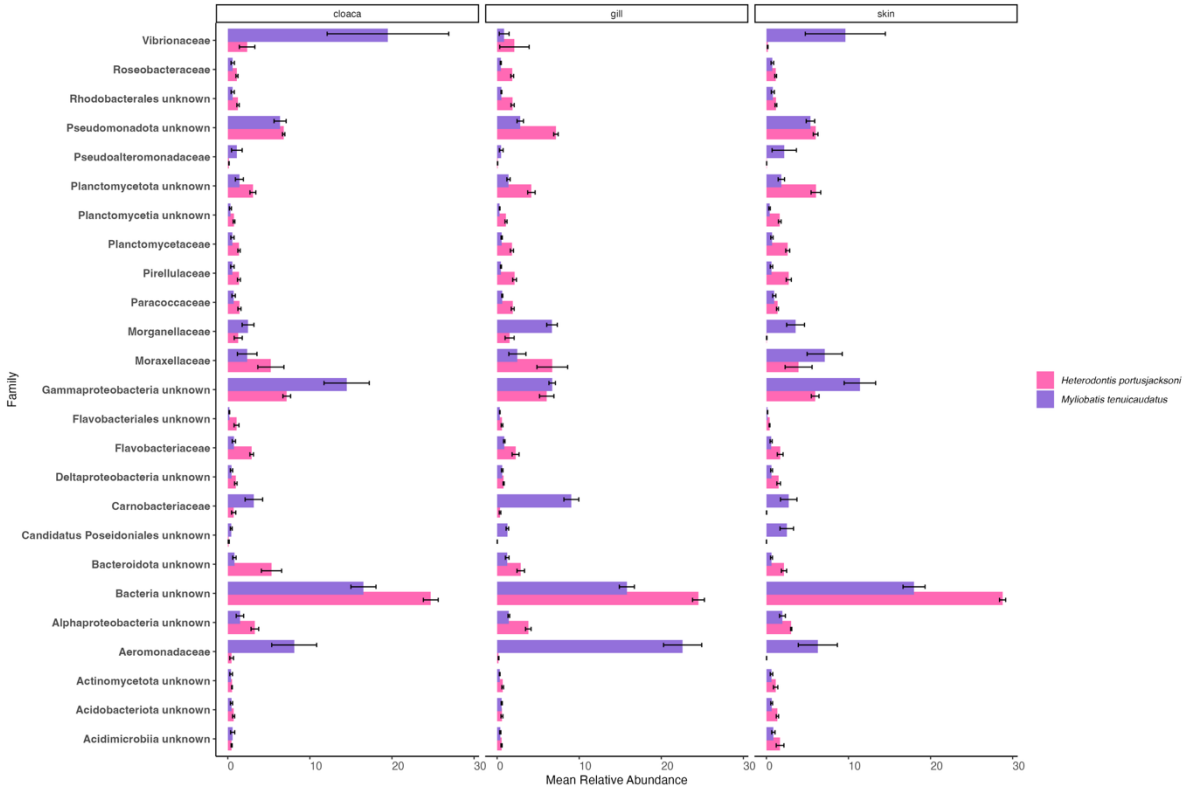

Figure 1.2: Mean relative abundance of abundant (at least 1% relative abundance) microbial families in *M. tenuicaudatus* and *H. portusjacksoni* microbiomes. Error bars represent standard error.

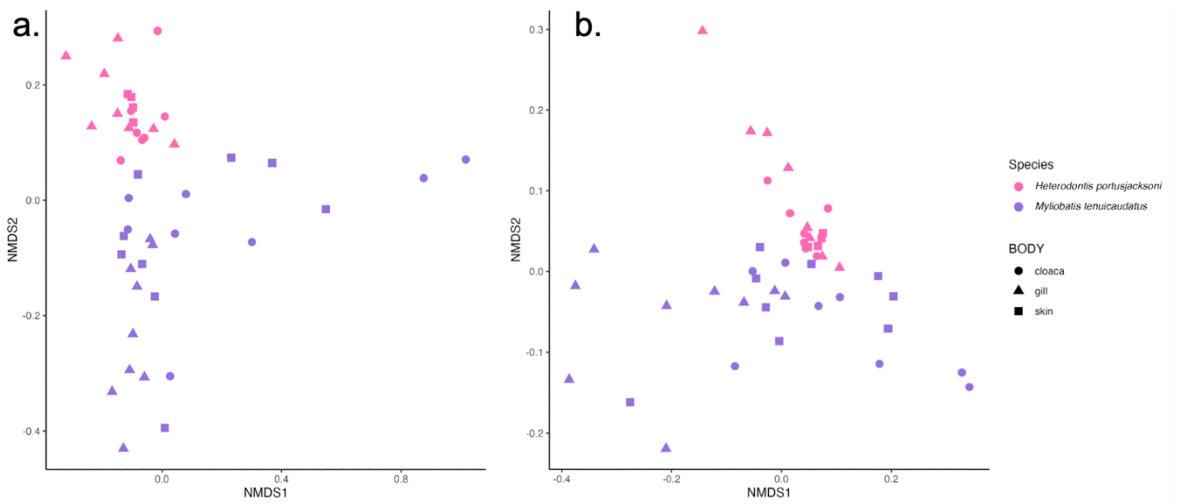

Figure 1.3: Nonmetric multidimensional scaling (NMDS) ordination calculated based on Bray–Curtis dissimilarity for a.) microbial family and b.) functional potential., Color denotes host species, and shape denotes body site sampled. High variability in *M. tenuicaudatus* taxonomic and functional communities, while *H. portusjacksoni* microbiomes cluster closely.

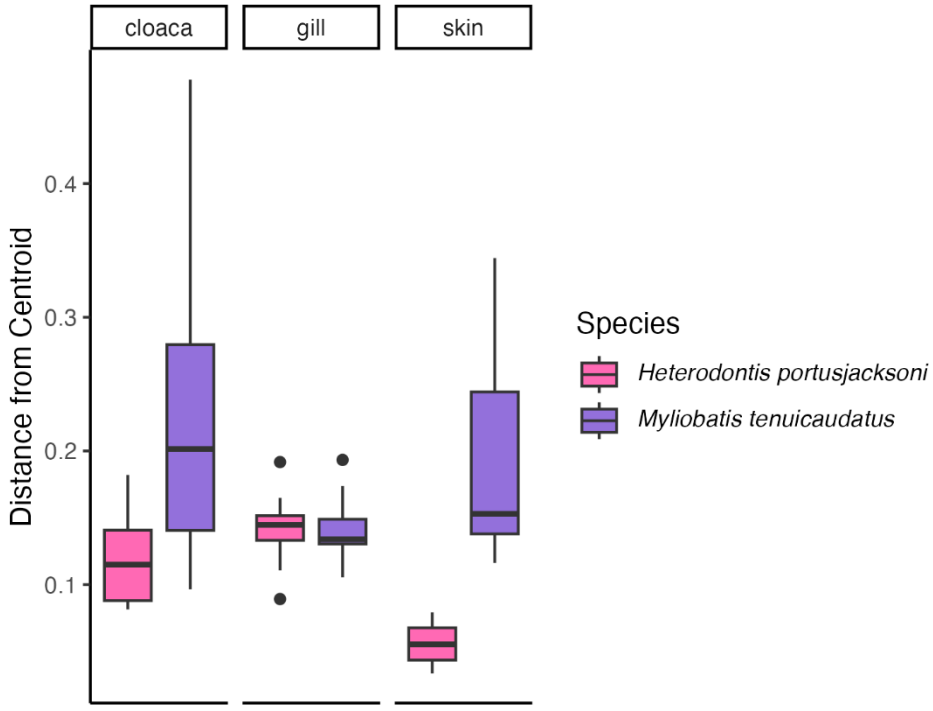

Figure 1.4: Dispersion (distance from centroid) for taxonomic communities of both hosts. *M. tenuicaudatus* had a higher and variable dispersion in cloaca and skin microbiomes compared with *H. portusjacksoni.* The gill microbiome of both species was highly consistent

across individuals. Centroids were calculated based on NMDS distances. Box and whiskers indicate inner quartile range.

Gill microbiomes across hosts

Host gills had significantly different microbial family and functional communities (PERMANOVA F = 12.11, df = 1, p = 0.001 and PERMANOVA F = 7.31, df = 1, p = 0.001). There were 15 bacterial families which were significantly different (LFC > 1 and p < 0.01) in shark and ray gill communities. *Aeromonadaceae*, *Carnobacteriaceae*, and *Morganellaceae* were more abundant in rays while unidentified Families in Pseudomonadota and Planctomycetota were more abundant in sharks (Figure 1.2).

Myliobatis tenuicaudatus gills had 13 functional genes that were significantly more abundant (LFC > 1 and p < 0.01), a majority of which belonged to protein synthesis, amino acid, and energy metabolism pathways (Supplementary Figure 1.3). Of the significantly different genes, lysine fermentation, metabolite repair and sporulation were most abundant. Within *M. tenuicaudatus* they each had a relative abundance of 4.2% 3.56 % and 3.11 % respectively while in *H. portusjacksoni* sharks these pathways had a relative abundance less than 0.5 % each. None of the differentially abundant genes had a higher abundance in *H. portusjacksoni* than *M. tenuicaudatus*.

Skin microbiomes across hosts

Pairwise comparisons showed *M. tenuicaudatus* and *H. portusjacksoni* skin had different family level communities (PERMANOVA F = 3.95, df = 1, p = 0.019) but not functional potential (PERMANOVA F = 2.27, df = 1, p = 0.087). Within the skin microbiomes, 194 families were differentially abundant (p < 0.05). *Candidatus*

Poseidoniales unknown and *Aeromonadaceae* were most different between species (Logarithmic Fold Change (LFC) > 8 and p adj = < 0.001). *Aeromonadaceae* had a higher average relative abundance in ray skin (7.46%) than in sharks (0.02%). *Candidatus Poseidoniales* had an average relative abundance of 1.7 % in *M. tenuicaudatus* and was extremely low in *H. portusjacksoni* (average relative abundance of 0.009%). Vibrionaceae (5.89%in *M. tenuicaudatus*, 0.16% in *H. portusjacksoni*), *Carnobacteriaceae* (3.10% in *M. tenuicaudatus* 0.02% in *H. portusjacksoni*), *Morganellaceae* (3.22% in *M. tenuicaudatus*, 0.04% in *H. portusjacksoni*), and *Pseudoalteromonadaceae* (1.78% in *M. tenuicaudatus*, 0.03% in *H. portusjacksoni*) were significantly more abundant (LFC > 2 and p < 0.05) in *M. tenuicaudatus* than in *H. portusjacksoni*. Abundant (greater than 1% average relative abundance) and significantly different (LFC > 2 and p < 0.05) families in sharks include an unknown *Planctomycetota* (6.03% in *H. portusjacksoni*, 2.13% in *M. tenuicaudatus*), unknown *Planctomycetia* (1.63% in *H. portusjacksoni*, 0.47% in *M. tenuicaudatus*) and *Planctomycetaceae* (2.60% in *H. portusjacksoni*, 0.83% in *M. tenuicaudatus*), *Pirellulaceae* (2.72% in sharks, 0.80% in *M. tenuicaudatus*) and unknown Bacteroidota family (2.15% in *H. portusjacksoni*, 1.19% in *M. tenuicaudatus*).

Metabolic functions functional gene pathways were present across the skin of both species. There were nine shared functional gene pathways in 1% abundance or greater in both host's skin microbiomes. These include Heme O and Heme A biosynthesis (2.4% in *H. portusjacksoni* and 2.9% in *M. tenuicaudatus*), Gram negative cluster (2. 1% in *H. portusjacksoni* and 1.5% in *M. tenuicaudatus*), and NADH ubiquinone oxidoreductase (1.5% in *H. portusjacksoni* and 1.82% in *M. tenuicaudatus*) (Supplementary Table 1.3).

Cloaca microbiomes across hosts

Cloaca of both species also had different family and functional microbiomes (PERMANOVA $F = 4.48$, $df = 1$, $p = 0.002$ and PERMANOVA $F = 4.03$, $df = 1$, $p = 0.008$). Nine families were differentially abundant ($LFC > 1$ and $p < 0.01$) across hosts. *Myliobatis tenuicaudatus* had a higer relative abundance of Vibrionaceae (19.51 %), an unknown Gammaproteobacteria clade (14.48 %) and Aeromonadaceae (8.09 %) than *H. portusjacksoni* (2.53 %, 7.18 % and 0.47 % respectively). *Heterodontus portusjacksoni* had significantly more Bacteroidota unknown (5.33 %) and *Flavobacteriaceae* (2.91 %) than *M. tenuicaudatus* (0.80 % and 0.74 % respectively).

Only four functional gene pathways had an average relative abundance of at least 1% and were significantly differentially abundant ($LFC > 1$ and $p < 0.01$) across hosts. Heme O and A biosynthesis was more abundant in *H. portusjacksoni* (7.21%) than in *M. tenuicaudatus* (2.10 %). Flagellum (1.17 %), metabolite repair (1.17 %) and sporulation cluster (1.11 %) were more abundant in *M. tenuicaudatus* than in *H. portusjacksoni* (0.50 %, 0.20 %, and 0.39 % respectively) (Supplementary Figure 1.4).

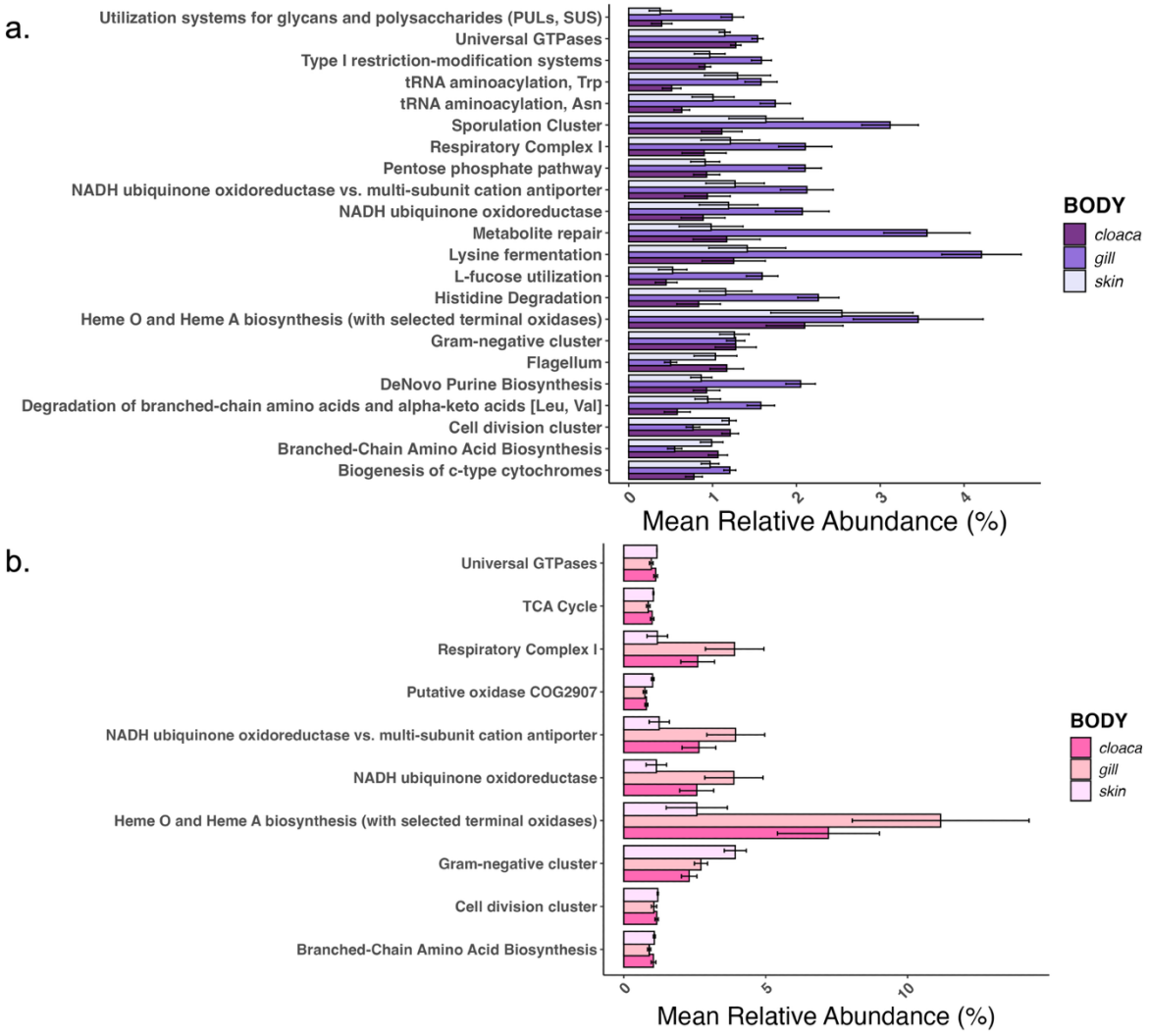

Figure 1.5: Abundant functional genes (> 1% relative abundance) for a.) *Myliobatis tenuicaudatus* and b.) *Heterodontus portusjacksoni* microbiomes body sites.

Metagenome Assembled Genomes

Five high quality Metagenome Assembled Genomes (MAGs) were recovered from *M. tenuicaudatus* assemblies (Table 1.2). One MAG belonging to the *Psychrobacter* clade was recovered from a *H. portusjacksoni* skin sample. Photobacterium MAGs assembled

from Pratte et al., 2022 (MAX001 and MAX005) and MAG22969 assembled from *M. tenuicaudatus* were compared to five reference genomes in BVBRC (Supplementary Table 1.2). Elasmobranch photobacterium MAGs contained five phage genes, a transposase gene, a sodium dependent transporter (*NupC*) gene, and seven hypothetical proteins that were not present in database representatives (Table 1.2). *Photobacterium damselae* MAG 22969 had five unique prophage regions (Figure 1.6). The prophages contained typical capsid and tail proteins and lacked virulence and antimicrobial resistance genes (Table 1.3). The prophages in MAG 22969 had no matches in the Sequence Read Archive (SRA).

Table 1.1: Metagenome assembled genome statistics

MAG	Species	Completeness	Contamination
22969	*Photobacterium damselae*	70.85	5.17
1590	*Vibrio diabolicus*	82.76	6.03
1591	*Vibrio alginolyticus*	84.48	1.88
23126	*Photobacterium leiognathi subsp. mandapamensis*	98.29	5.17
6425	*Psychrobacter cryohalolentis*	80.46	2.59

Table 1.2: Genes exclusive to Elasmobranch *P. damselae* and genes exclusive to database isolates. Hypothetical genes are not included. ER22969 originated from *M. tenuicaudatus* cloaca, MAX001 and MAX005 were assembled from *R.typus* and *C. carcharias* cloaca co-assemblies. Other

Gene	ER22969	MAX001	MAX005	AS-15-3942-9	AS-16-0963-3	KC-DI-1	206352-6(DK32)	80077637	A-162

Transposase	X	X	X						
Na+ dependent nucleoside transporter NupC	X	X	X						
Phage baseplate assembly protein J	X	X	X						
Phage tail fiber protein	X	X	X						
Phage tail length tape-measure protein T	X	X	X						
Phage teminase, large subunit	X	X	X						
Phage minor tail protein Z	X	X	X						
FIG011065: hypothetical protein				X	X	X	X	X	X
Primosomal replication protein N prime prime				X	X	X	X	X	X
Uncharacterized protein YbaM				X	X	X	X	X	X
Outer membrane beta-barrel assembly protein BamE				X	X	X	X	X	X
UPF0125 protein RatB				X	X	X	X	X	X
Periplasmic protein SypC involved in polysaccharide export				X	X	X	X	X	X
Anti anti-sigma regulatory factor SypA				X	X	X	X	X	X
Sugar transferase SypR involved in lipopolysaccharide synthesis				X	X	X	X	X	X
Glycosyltransferase SypN				X	X	X	X	X	X
Membrane protein SypL involved in exopolysaccharide production				X	X	X	X	X	X
Oligosaccharide translocase SypK				X	X	X	X	X	X
Protein of unknown function DUF1282				X	X	X	X	X	X
NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)				X	X	X	X	X	X
Anaerobic respiratory reductase, anchor subunit				X	X	X	X	X	X
Hydroxymethylpyrimidine ABC transporter, ATPase component				X	X	X	X	X	X
Uncharacterized protein YjqA				X	X	X	X	X	X
NTP pyrophosphohydrolases including oxidative damage repair enzymes				X	X	X	X	X	X

GlpM protein	X	X	X	X	X	X
FIG00921131: hypothetical protein	X	X	X	X	X	X
Choloylglycine hydrolase (EC 3.5.1.24)	X	X	X	X	X	X
Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26)	X	X	X	X	X	X
FIG01289198: hypothetical protein	X	X	X	X	X	X

Table 1.3: Phage genes (annotated by phold) found in the five prophages from the *P. damselae* MAG originating from *M. tenuicaudatus* metagenome.

Description	Phage 1	Phage 2	Phage 3	Phage 4	Phage 5
CDS	38	16	11	22	31
connector	2	0	0	0	5
DNA, RNA and nucleotide metabolism	2	0	0	6	0
Head and packaging	7	10	3	0	5
Integration and excision	0	0	0	0	0
Lysis	1	0	0	1	2
Moron, auxiliary metabolic gene and host takeover	0	0	0	0	0
Other	2	0	1	0	0
Tail	11	4	2	0	14
Transcription regulation	1	0	0	2	0
Unknown function	12	2	5	13	5
VFDB Virulence Factors	0	0	0	0	0
CARD AMR	0	0	0	0	0
ACR anti crispr	0	0	0	0	0
Defensefinder	0	0	0	0	0
Netflax	0	0	0	0	0

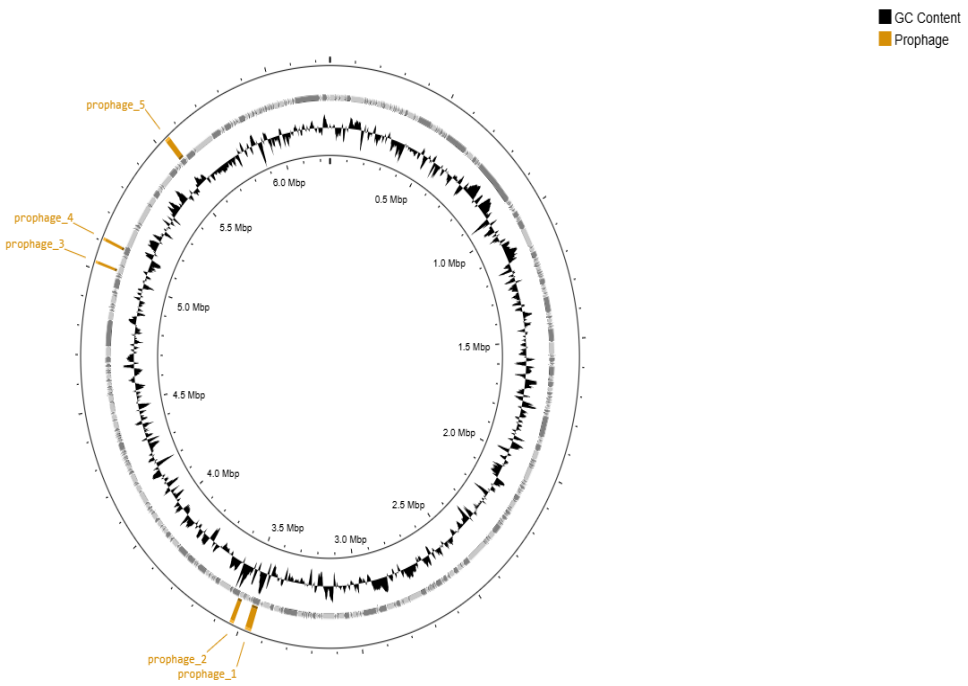

Figure 1.6: Genome visualization of MAG 22969. Highlighted are the five prophage regions.

Discussion

We explored the skin, gill, and cloaca microbiomes of two co-occurring host species to determine eco-evolutionary relationships between hosts and microbes at these body sites. The two Elasmobranch host species had distinct microbial taxonomic and functional communities at all body sites except the skin which were metabolically similar, suggesting that the host influences microbiomes on all surfaces, but skin microbes must also be functionally adapted to the surrounding water column. We propose that *Photobacterium damselae* is an Elasmobranch specific microbe with conserved prophage regions found globally. The ubiquity of *P. damselae* in Elasmobranch gut microbiomes across clades and environments suggests it is a symbiotic gut bacterium despite being a well-known

opportunistic pathogen in fish (Matanza et al., 2020). Prophage genes were present in the *P. damselae* MAGs originating from Elasmobranchs and absent in database *P. damselae* strains. Host morphology, including the amount of mucus and presence of dermal denticles appeared to structure the microbiomes of the cloaca and skin, but environmental influence was evident. Body sites with more mucus, the skin and cloaca of the *M. tenuicaudatus,* had higher variability than *H. portusjacksoni* where the microbiome was high similarity. The microbes associated with the gills, however, had reduced variation in both hosts, showing high similarity between individuals, indicating host is a major determinate of gill microbiomes.

Microbial communities of *Myliobatis tenuicaudatus*

Myliobatis tenuicaudatus body-site microbial communities had similar Hill diversity but gills had significantly different taxonomic composition to skin and cloaca communities (Figure 1.3a). Gills also had the least dispersion compared to skin and cloaca (Figure 1.4). Gills were dominated by *Aeromonadaceae,* a gram-negative facultative anaerobe (Martinez-Murica 2013). *Aeromonadaceae* are not commonly observed in high abundance in Elasmobranch microbiomes and have been identified as potential shark pathogens (Juste-Poinapen et al., 2019). The prevalence of *Aeromonadaceae* across samples of seemingly healthy individuals suggests they are adapted to living in the mucus layer of *M. tenuicaudatus* which is likely a low oxygen environment rather than acting as pathogens.

Interestingly, *M. tenuicaudatus* skin and cloaca were not significantly different in microbial family composition. High variation in skin and cloaca communities could be obscuring differences between these body sites (Figure 1.3, 1.4). Rays tend to have high variability in their microbiomes in both alpha and beta diversity measures (Clavere-Graciette et al., 2022, Kerr et al., 2023). In Elasmobranchs, cloaca and skin microbiomes are not

always different from one another, while differences were observed between other body sites (especially teeth and gills) (Storo et al., 2021). High relative abundance of families within the Vibrio clade were observed in both skin and cloaca samples of two individual rays. *Myliobatis tenuicaudatus* skin and cloaca microbiomes had a high relative abundance of Moraxellaceae and undescribed family of Gammaproteobacteria clade, and *Vibrionaceae* (Figure 1.4a). *Vibrionaceae* and *Moraxellaceae* are common bacteria in microbiomes of both wild and aquarium housed rays, suggesting they are host specific and unlikely to be pathogens (Clavere-Graciette et al., 2022; Kearns et al., 2017; Kerr et al., 2023; Pinnell et al., 2021). Although the effect of microbial communities at one body site on the microbial community at another within an individual has not yet been explored in sharks, it is unlikely that they act entirely independently from one another. In fish, skin and gill microbiomes change between disease state, and have been hypothesized to be indicators of gut health (Legrand et al., 2018) although this hasn't been addressed directly.

Myliobatis tenuicaudatus gill microbiomes had significantly different functional potential compared to skin and cloaca (Figure 1.3b). Functional genes that were abundant in gills include Lysine fermentation and metabolite repair (Figure 1.5). Lysine fermentation produces L-lysine, an essential amino acid (Yao et al., 2001). Host organisms can absorb lysine produced by microbes in the gut contributing to host health (Ye et al., 2023). The extent to which microbial metabolites is absorbed in the gills is unknown but given the importance of waste exchange in the gills (Evans et al., 2005) the exchange of other chemicals at this body site are likely important to host health. Metabolite repair was also abundant in gills (Figure 1.5). Metabolite repair enzymes minimize damage from toxic side products produces from normal cellular functioning (Bommer et al., 2020). The high relative

abundance of metabolite repair suggests that metabolites (waste) excreted by the host could be neutralized by symbiotic gill microbes.

Myliobatis tenuicaudatus cloaca and skin microbiomes had a high abundance of cell division and flagella genes. Mucus provides nutrients for symbiotic microbes (Kerr et al., In review), allowing for high growth rates (Sicard et al., 2018). Cell division genes enable bacterial populations grow quickly in high nutrient environments (Myhrvold et al., 2015; Ouwerkerk et al., 2013). Flagella genes were highly abundant in these body sites and are a key characteristic of motile bacteria and allow bacteria to travel through mucus environments like the gut (Paone & Cani 2020). The level of variability in the skin and cloaca microbiomes are consistent with high turnover rates of the mucus (Burgess et al., 2018) which is not observed in shark samples.

Microbial communities of *Heterodontus portusjacksoni*

Heterodontus portusjacksoni had significantly different family communities at each body site (Figure 1.3a) but Hill diversity was maintained. This is consistent with other species of shark including black tip reef sharks (*C. melanopterus*) which also demonstrate distinct skin, oral and cloaca communities (Pogoreutz et al., 2024). *Moraxellaceae* was the most abundant bacteria identified to the family level and is present in other shark microbiomes (Black et al., 2021; Bregman et al., 2022). Other clades of typical shark associated microbes including Gammaproteobacteria were present but could not be identified at the family level. *Heterodontus portusjacksoni* have a high proportion of novel bacteria (Figure 1.2) and require further characterization.

Heterodontus portusjacksoni functional potential was similar across all body sites tested (Figure 1.3b). Predicted metabolic functions of *C. melanotropes* skin included

glycogen and aromatic compound degradation and cloaca included genes for sulfate and galactose degradation (Pogoreutz et al., 2024). Glycogen metabolism and galactose degradation genes in skin microbiomes of Elasmobranchs are implicated in the metabolism of skin mucus (Kerr et al., In review). *Carcharhinus melanopterus* microbiomes had fewer than 20 overrepresented predicted pathways in each tissue microbiome, suggesting functional gene similarity despite taxonomic differences similar to *H. portusjacksoni* (Pogoturez et al., 2021). Mucus chemistry may be conserved across body sites (Thomsson et al., 2025), and therefore microbes require similar genes to breakdown mucus in the gut and on the skin, leading to similarities in functional potential across body sites.

Gill microbiomes across hosts

Gill microbiomes were the most distinct from other body sites and had the lowest dispersion in both species (Figure 1.4). Gills are sensitive organs which can be routes of respiratory infections (Koppang et al., 2015). Because of this host immune systems may play a larger role in regulating microbial communities than other body sites. In *Dascyllus abudafur*, a coral reef fish, skin and gut were more influenced by coral colony probiotics than gills. Gills did not have any potentially pathogenic taxa increase significantly in abundance between treatments, when skin and gut did (Rosado et al., 2025). Low dispersion suggests that gill microbiomes in *M. tenuicaudatus* and *H. portusjacksoni* are regulated by the host and are less affected by environmental fluctuations than skin or cloaca.

Teleost fish gills often have a high relative abundance of Nitrosomonas (family *Nitrosomonadaceae*) in gill microbiomes, hypothesized to metabolize nitrogen waste excreted by the host (Legrand et al., 2018). *Nitrosomonadaceae* accounted for less than 0.01 % of gill microbiomes in either host and ammonia oxidation genes were similarly low in abundance. Elasmobranchs can re-absorb ammonia through the gills, which might reduce

the amount available for microbes to metabolize (Perry et al., 2021). Lack of nitrogen metabolism in the gill microbiomes suggests gill bacteria in Elasmobranchs are performing different roles than those in fish gills, but both fish and shark gill microbiomes are highly specific and regulated by the host.

Skin microbiomes across hosts

Skin taxonomic microbiomes of both species were significantly different (Figure 1.3a). *Vibrionaceae* and *Aeromonadaceae* were significantly more abundant in *M. tenuicaudatus* (7.5 and 5% respectively) and were almost absent in *H. portusjacksoni* (less than 0.05%) (Figure 1.2). Vibrio are common stingray associated bacteria (Clavere-Graciette et al., 2022; Kerr et al., 2023) and have a high relative abundance particularly in wild rays (Pinnell et al., 2021). Planctomycetota clades were significantly more abundant in *H. portusjacksoni* than *M. tenuicaudatus* (Supplementary Figure 1.1). Planctomycetota have diverse metabolisms including novel carbohydrate-active enzymes (CAZymes) (Klimek et al., 2024) which could indicate this is a generalist taxon adapted to the host and surrounding water column.

Despite taxonomic differences, both hosts had similar skin microbial functions. This suggests that microbes are adapted to the skin of the host but also impacted by their shared environment. Amino acid biosynthesis and TCA cycle were both among the most abundant gene functions in both hosts. Other shared pathways were also “housekeeping” genes universally utilized by bacteria for basic cell functions like DNA replication and respiration (Joshi et al., 2022). The similarly of gene pathways in skin suggests skin bacteria are more generalist than those found at other body sites. While those generalist genes are present in other body sites, an increase in the presence of more specific gene pathways like flagella or

lysine fermentation shows body site-specific adaptations of internal body-site communities, driven by host and less by the environment.

Cloaca microbiomes across hosts

Cloaca family and functional potential varied between *M. tenuicaudatus* and *H. portusjacksoni*. *Vibrio* species, specifically *Photobacterium damselae*, were significantly more abundant in *M. tenuicaudatus* than *H. portusjacksoni. Heterodontus portusjacksoni* had significantly more *Flavobacteriaceae*, which is a common gut bacterium in Elasmobranchs (Clavere-Graciette et al., 2022; Leigh et al., 2021; Pinnell et al., 2021; Pratte et al., 2022). Differences in gut communities between the two hosts indicate these are more influenced by host than environment. Common Elasmobranch gut bacteria also indicate host phylogeny also shapes gut bacteria, and indicator of phylosymbiosis (Doane et al., 2020).

Despite differences in overall composition, only four functional pathways were significantly different between hosts; Heme O and A biosynthesis was more abundant in *H. portusjacksoni.* Heme O and A are required for aerobic respiration (Rivett et al., 2021) which may be a characteristic of a low mucus (and therefore high oxygen) environment. Metabolite repair, and flagellum were significantly more abundant in *M. tenuicaudatus.* Metabolite repair is a broad category of enzymes that remove or modify metabolites produced directly or as side products of other chemical reactions (Galperin et al., 2006). Metabolite repair could play in important role in digestion and utilization of compounds metabolized by other members of the community (Bommer et al., 2020). Flagella are required for motility in high mucus environment like ray mucus (Moens & Vanderleyden, 1996). While few genes were significantly overrepresented, overall differences in the functional potential and specific genes indicate that bacteria are specifically adapted to the gut of each species.

Myliobatis tenuicaudatus vs. *Heterodontus portusjacksoni* microbiomes

Overall, host had significantly different family and functional communities (Figure 1.3), except the skin which shared similar functional potential., Shared functional genes and distinct taxonomy in skin microbiomes indicates hosts are selecting different microbial taxa but those taxa must be adapted to the host and surrounding environment. In other body sites (gills and cloaca) host plays a larger role in structuring microbial communities at taxonomic and functional scale.

Elasmobranch microbiomes tend to vary between species (Clavere-Graciette et al., 2022; Goodman et al., 2024; Kerr et al., 2023) which is supported here, even when collected from similar environments. Dispersion was higher in *M. tenuicaudatus* compared to *H. portusjacksoni* (Figure 1.4) which is characteristic of rays and could be attributed to a stronger environmental influence on ray microbial communities (Clavere-Graciette et al., 2022; Kerr et al., 2023; Pinnell et al., 2021) likely due to mucus turnover. Mucus responds to changes in the environment like temperature, pH, and dissolved gasses in addition to being sloughed as the rays move through the water (Cabillon & Lazado, 2019; Parrish and Kroen 1988). Changes and losses in surface mucus due to change in environmental conditions could be disrupting microbes causing variability in ray microbiomes. When comparing wild and captive rays, skin and gill diversity tends to be lower in captive groups, possibly due to reduced microbial diversity in aquarium water (Clavere-Graciette et al., 2022; Pinnell et al., 2021). *Triakis semifasciata* however, maintain diversity in captivity (Goodman et al., 2022) indicating shark and ray microbiomes react to changes in the environment differently. *Heterodontus portusjacksoni* had less intraspecific variation than *M. tenuicaudatus* (Figure 1.4). Few physiological changes have been observed in benthic sharks in response to ocean acidification, but behavioral changes have been observed (Rosa

et al., 2017). In low-salinity, leopard sharks do adapt physiologically, but not all metabolic changes were significantly different (Dowd et al., 2010). *Heterodontus portusjacksoni* under end-of-century climate conditions showed little physiological plasticity regarding muscle fiber size and density (Thomas et al., 2023). This suggests that while some shark species can tolerate environmental differences, physiological response may be delayed or minimal., Therefore, microbes may not have large physiological changes to adapt to in sharks leading to a less variable microbial community overall. The skin microbiome of sharks has low intraspecies variation which is hypothesized to be due to denticles selecting and excluding microbes from the environment (Doane et al., 2017, 2020). Denticles contribute to structuring skin communities, but other chemical or microbial interactions could also be driving the functional similarity in other body sites. *Carcharhinus melanopterus* had different microbial communities at external body sites driven by a high number of rare taxa (Pogoreutz et al., 2024). In *H. portusjacksoni* microbes may be adapted to the host overall and functions that are different across body sites are in low relative abundance.

Metagenome Assembled Genomes

Five MAGs were assembled from the dataset, four Vibrio species and one Psychrobacter species (Table 1.2). Only one MAG was assembled from *H. portusjacksoni* which belonged to Psychrobacter clade. *Heterodontus portusjacksoni* microbiomes were extremely diverse at all body sites which is a challenge for existing assembly and binning tools (Papudeshi et al., 2017). *Photobacterium damselae* was assembled from a cloaca sample from *M. tenuicaudatus* (Figure 1.6). *Photobacterium damselae* has been identified in gut or cloaca samples of *C. carcharias*, and *R. typus*, and is highly abundant in *Sphyrna tiburo*, *Aetobatus narinari*, *Rhinoptera bonasus, Urobatis jamaicensis, Carcharhinus melanopterus, Carcharhinus plumbeus, Rhizoprionodon terraenovae*, *Carcharhinus*

brevipinna, spanning different locations, diets, life history, and captive status (Clavere-Graciette et al., 2022; Givens et al., 2015; Leigh et al., 2021; Pinnell et al., 2021; Pogoreutz et al., 2024; Pratte et al., 2022). This ubiquity of *P. damselae* in shark gut microbiomes and the conserved prophages suggests this strain is a critical gut bacterium.

I compared the genes present in Elasmobranch MAGs to references in the database originating from humans, fish and marine mammals (Supplementary Table 1.2). *Photobacterium damselae* has been assembled from shark gut samples previously (Pratte et al., 2022) and when compared with MAG22969, shared seven hypothetical protein genes, five phage genes, one transposase gene and one sodium dependent transporter gene (*NupC*) which were absent in database strains (Table 1.2). Presence of specific prophage genes in only shark-derived *P. damselae* suggests that prophages could be playing a role in preventing this species acting as a shark pathogen. When viruses that infect bacteria (bacteriophages) incorporate DNA into the host genome, they are called prophages (Canchaya et al., 2003). When, induced, prophages begin to replicate forming hundreds of bacteriophages which lyse the bacterial cell. Prophages regulate bacterial populations when induced but can also improve survival of the host by introducing genes into the bacterial genome (Inglis et al., 2024; Knowles et al., 2016). Prophages and transposons were abundant in *R. typus* faecal microbiomes (Pratte et al., 2022), further supporting the importance of prophages in the gut of sharks. Previous investigations of shark bacteriophages are limited to the skin (Hesse et al., 2023) and even in human gut microbial communities, prophages are underexplored (Inglis et al., 2024). Future investigation of the role of bacteriophage in shark guts would confirm their role in the shark gut microbiome.

Conclusion

I compared two hosts with similar ecology but distinct physiology at three body sites. I found that *M. tenuicaudatus* gill microbial communities were distinct from skin and cloaca in family and functional composition, driven by high variability in skin and cloaca due to thick mucus. *Heterodontus portusjacksoni* had distinct family communities at all body sites but had similar functional potential., This suggests that microbes are uniquely adapted to this shark species, regardless of body site. When comparing the two hosts, microbial families and functions were different across all body sites except skin functions were similar, indicating an environmental influence on skin functions more than other body sites. Microbes on the skin may be generalists and remain adapted to the changes in the environment. Host internal body sites (gill and cloaca) were most distinct between host species in both taxonomy and functional potential suggesting they are more influenced by host factors than the environment. I assembled a *Photobacterium damselae* MAG with Elasmobranch specific prophage genes. This bacterial species is conserved across Elasmobranch hosts and likely plays a major role in the gut microbial community, regulated by prophage.

CHAPTER 2: JUVENILE ELASMOBRANCH MICROBIOMES ARE INFLUENCED BY BUT DISTINCT FROM THE ENVIRONMENT

Context

Elasmobranch skin is characterized by dermal denticles and mucus which protects the organism from the environment. Denticles and mucus directly influence the microbial community of adults, playing a role in differentiating host microbiomes from one another. Microbiomes during embryonic development of skates showed differences through development and between juveniles and adults (Mika et al., 2021). No other juvenile Elasmobranchs have been investigated previously. To investigate the influence of skin structure and season on juvenile Elasmobranch microbiomes I collected skin microbiomes from four hosts species over two sampling events. This work is being prepared for submission to Ecology and Evolution.

Abstract

Juvenile Elasmobranchs use shallow protected habitats as nursery areas. These environments are subject to large shifts in conditions due to their shallow nature and proximity to the coast. To test the impacts of environmental change on mucus or denticle microbiomes I collected skin microbiomes from eight species of Elasmobranch in the Gulf St. Vincent, South Australia. Samples were collected opportunistically during two SARDI observer trawling operations in March and April 2023, resulting in 68 metagenomic samples. Elasmobranch skin microbiomes were distinct from the surrounding water column, suggesting host skin whether mucus or denticle dominated is selective even as juveniles. Interestingly, hosts did not have significantly different family composition regardless of skin morphology. There was however a noticeable shift in microbial communities of all hosts between sampling periods (approximately 30 days). March had the highest variation in temperature and the most

variation across all host microbiomes. April has more stable and colder temperatures and the least dispersion in microbial communities. Similar shifts in microbiome structure in both mucus and denticle skin types suggests that the environment affects both microbial taxa and functions in juvenile Elasmobranch microbiomes. Similar shifts across all hosts indicate conserved response to environmental change which may help hosts adapt to changes in environmental conditions.

Introduction

Microbiomes are dynamic communities of microorganisms which interact with each other and their host (Berg et al., 2020). Host associated microbial communities are essential for health, contributing to immune functioning and metabolism, and are influenced by the surrounding environment (Sehnal et al., 2021). In dynamic inner reef environments, coral microbiomes fluctuate, while in stable outer reef habitats microbial communities are more stable (Lima et al., 2022). In *Scomber japonicus* (pacific chub mackerel) fish age, chlorophyll, and temperature significantly influenced microbiome diversity (Minch et al., 2020). Juvenile Atlantic salmon (*Salmo salar*) skin and gill microbiomes reflected lower bacterial diversity in the water column (Lorgen-Ritchie et al., 2022).

Sheltered coastal habitats such as estuaries (and reverse estuaries) are utilized by a variety of fish and sharks as nursey grounds (Unsworth et al., 2018, Whitfield et al., 2017). Young sharks exhibit high site fidelity, sometimes remaining in seagrass nurseries for up to two years (Powter et al., 2009). Protection of nursery habitats contribute positive conservation outcomes for species with high residency (Kraft et al., 2024), but damage to these habitats make juveniles more susceptible to the effects of climate change (Niella et al., 2022). Anthropogenetic stressors including pollution, habitat degradation, and climate

change all negatively affect Elasmobranch reproduction and development (Wheeler et al., 2020).

The Gulf St Vincent is a 13000 km^2 basin off the coast of South Australia (Shepard and Spriggs 1976). The seagrass, sponge garden, and soft bottom habitats make up a large proportion of biodiversity in the region (Edyvane 1999). The region has high Elasmobranch biodiversity with over 40 species occupying the gulf (Baker et al., 2008). Tourism and fisheries in the Gulf St. Vincent ecosystem are also important contributors to the local economy (Twidale, Tyler & Webb 1976). In recent decades, the Gulf St. Vincent has experienced a decrease in habitat complexity due to trawling and increase in anthropogenic activity (Leterme & Tuuri et al., 2023; Tanner et al., 1998). The Gulf St. Vincent tends to have higher salinity than the ocean due to high evaporation rates and low rainfall input, especially in summer months (de Silva Samarasinghe et al., 2003).

Given the Gulf St. Vincent's unique biogeographical features and Elasmobranch diversity, I investigated the microbiomes of six species of Elasmobranch, *Squatina australis, Trygonorrhina dumerilii, Notorynchus cepedianus, Dentiraja cerva, Heterodontus portusjacksoni,* and *Aptychotrema vincentiana*. I compared microbiome taxonomic and functional composition and microbial abundance of four of the host species (*T. dumerilii*, *D. cerva*, *S. australis*, *N. cepedianus*) across two sampling events approximately 30 days apart (Table 2.1). I construct novel Metagenome Assembled Genomes from host and water column metagenomes.

Methods

Sample Collection

Elasmobranchs were captured opportunistically during scientific survey for the commercial prawn trawling industry in the St Vincent Gulf, South Australia, Australia. On these trawls the exclusion device was removed from one of the nets, providing access to Elasmobranch bycatch. During the prawn season however, the boats use exclusion devises on both nets. Trawls were deployed at depth for 30 minutes (McLeay & Hooper 2024; Burnell et al., 2015). Upon retrieval, Elasmobranchs were separated from the other organisms and placed in a tub filled with fresh seawater, which was replenished between trawls. Organisms were gently "supersukered" where a blunt-end-two-way syringe filled with sterile seawater was pressed against the dorsal skin to create a seal., The syringe was depressed, flushing the skin with sterile seawater dislodging microbes which were then recollected in the body of the syringe. This was repeated four times which yields about 200 mL of microbial slurry from each organism. From each sample 1 mL was added to a cryovial with 20 µL of 25% glutaraldehyde and stored in the dark at 4°C. The remaining sample was filtered through a 0.22 µm Sterivex filter. The size and sex of each Elasmobranch was measured. Measurements included total length for sharks and disk diameter for rays. Once onshore, microbial samples were transported on ice then stored at -20ºC until DNA extraction. Water samples were collected from across South Australia (Table 1). At several sites 1-2 litres of water was filtered through a 0.22 µm Sterivex filter. For flow cytometry samples, 1 mL of seawater was preserved with 20 µL of 25% glutaraldehyde and stored in the dark at 4°C. Environmental data (sea surface temperature, chlorophyll a concentration) was sourced from Australia's Integrated Marine Observing System (IMOS) through the Australian Ocean Data Network (AODN).

Metagenomics

Macherey-Nagel Nucleospin tissue kit was used to extract DNA directly from Sterivex filters (Macherey-Nagel, Germany). Briefly 720 µL of T1 buffer and 90 µL of Proteinase K were added to each filter and incubated with rotation overnight. After incubation, the contents of each Sterivex were spun into 5 mL tubes (Eppindorf, Australia). 800 µL of B3 buffer was added to the lysate and incubated for 10 minutes at 70ºC. 800 µL of 100% ethanol was added and samples were shaken gently. 550 µL of each sample was loaded onto a spin column and spun at 14000 rpm for one minute, flow through was discarded. This step was repeated 5-6 times until the entire sample was loaded onto the column. Once the sample was loaded, 500 µL of BW buffer was added and spun at 14000 rpm for one minute and flow through was discarded. B5 buffer (600 µL) was added, spun at 14000 rpm for one minute and flow through was discarded. Filters were spun dry for two minutes at 14000 rpm. Spin columns were transferred to sterile 1.5 mL Eppendorf tubes. BE buffer (50 µL) was added and incubated at room temperature for ten minutes then spun at 14000 rpm for 1 minute. This step was repeated to yield 100 µL of eluted DNA. DNA concentration was quantified with the Qubit flurometer (Invitrogen) then stored at -20ºC.

Libraries were prepared using the IDT xGen™ DNA Library Kit which utilizes, mechanically sheared DNA. Samples were diluted in thin-walled PCR tubes to 2 ng/µL in 50 µL if possible. If the concentration of a sample was less than 2 ng/µL, 50 µL undiluted sample was used for sonication. Samples were sonicated using the QSonica Q800R model (QSonica Sonicators, USA). Samples were sonicated at 20% amplitude for 9 minutes with alternating sonication periods of 15 seconds of sonication followed by a 15 pause. Samples were sonicated at 4°C. Post sonication, libraries were prepped with the xGen™ DNA Library Kit following manufacturer instructions for 300bp input fragments. The final

indexing step uses a 12 cycle PCR (Integrated DNA Technologies, USA). Libraries were sequenced by the Australian Genome Research Facility on the Illumina NexSeq (Illumina, USA).

Bioinformatics

Fastq files were quality controlled, annotated and assembled using the atavide-lite pipeline (https://github.com/linsalrob/atavide_lite). First, quality control is implemented with Fastp (Chen 2023) to remove adaptors, and discard reads with more than one ambiguous base (N) and sequences less than 100 bp in length. Quality controlled sequences are available on the Sequence Read Archive under BioProject PRJNA1245075. Host DNA was then estimated by mapping reads with minimap2 (Li et al., 2018) to bespoke database of all publicly available Elasmobranch genomes and reads that match Elasmobranch were removed. Taxonomy and functional gene annotations are carried out using mmseqs (Steinegger & Soding 2017) against the UniRef50 database (Suzek et al., 2007). Each sample was assembled with Megahit (Li et al., 2015). The resulting contigs longer than 1000 bp were binned using VAMB (Nissen et al., 2021). Unique genomes in the dataset were identified using dRep (Olm et al., 2017). CheckM was used to assess MAGs for completeness and contamination (Parks et al., 2015). All bioinformatics analysis was completed using the Flinders University HPC Deepthought (Flinders University). Only high-quality bins with 70% completeness or greater and less than 10% contamination are included in this analysis (Papudeshi et al., 2017). MAGs were annotated with BVBRC Genome Annotation tool. Phylogenetic tree of all MAGs was constructed using the BVBRC Bacterial Genome Tree tool with 50 genes selected for alignment, a maximum of 10 deletions and 10 duplications allowed (Olson et al., 2022).

Flow Cytometry

Microbial counts were performed using flow cytometry with the CytoFlex S and SRT. A template was created for analyzing microbial and viral particles using the Small Particles II configuration. 5 µL of the initial fixed sample was diluted in 495 µL of 0.22 µm-filtered Tris EDTA buffer (1:100 dilution) and stained with 12.5 µL of SYBR Green II, then incubated at 70ºC for 15 minutes. Samples were calibrated for size using 10 µL of 1 µm fluorescent beads suspended in MiliQ water added immediately before processed in the cytometer for 2 minutes at a rate of 31 µL/sec. Cytometry data was analyzed using FlowJo v10.9.0.

Statistics

Statistical analyses were conducted in RStudio (V 2024.12.0) using base R (V 4.4.2) (R Core Team 2024) and Primer7 (V.7.0.21) (Primer-E, UK). Eukaryotic and viral reads were excluded from the analysis. An unknown Bacteria clade was highly abundant (18-25%) across host and water microbiomes. Reads from this clade was removed from analysis and relative abundance was recalculated to avoid this group of unknown taxa from obscuring the influence of less abundant known taxa in the dataset. Good's coverage (number of families present only once relative to the total) was calculated for each sample to ensure adequate coverage was achieved (98% or higher for all samples). All samples had greater than 15,000 reads and 98 % or higher Good's coverage and were included in analysis. Relative abundance for each family was calculated by taking the number of reads in each family divided by the total reads in the sample multiplied by 100 using the tidyverse and dplyr packages (Wickham et al., 2019, Wickham et al., 2023). To compare microbiome taxonomic and functional composition across sharks and the water column, data was square root

transformed Bray-Curtis distances were calculated using Primer7 (Primer-E, UK). Bray-Curtis distances were visualized as NMDS plots using ggplor2 (Oksanen et al., 2024). Differences in microbiome composition between the water column and hosts and across hosts and month sampled were tested using PERMANOVA in Primer7 (Primer-E, UK). Microbiome variation was tested using Permutation test for homogeneity of variance (PERMDISP) in the vegan package (Wickham et al., 2016).

Hill diversity was calculated using vegan and visualized with boxplots using ggplot2 (Wickham et al., 2016). Kruskal-Wallis was used to compare host microbiome diversity for host, month and sex (where sample size allowed) using the R 4.4.2 (2021).

DSeq2 was used to compare the taxa and functions that differed significantly between March and April sampling dates (Love et al., 2014). Taxa that were differentially abundant (greater than 2 log fold change and $p < 0.01$) between March and April and had an average relative abundance of at least 1% in were plotted as a group bar chart using ggplot2 (Wickham et al., 2016). Level 3 functions that were significantly different between March and April (greater than 2 log fold change and $p < 0.01$) and had a relative abundance of at least 0.01% were plotted.

Bacterial abundance was compared between each host and month using a Kruskal-Walis test. Due to low sample size water column bacterial abundance could not be tested between months or compared to hosts statistically.

Table 2.1: Summary of sample size, average host size (± standard error), and host sex.

Host	Month		Total length (cm)	Disc width (cm)	Fork length (cm)	Sex	
	March	April				Female	Male
Squatina australis	9	3	38 ± 3.0	23 ± 1.9		10	2
Trygonorrhina dumerilii	11	10	81 ± 4.5	36 ± 1.7		10	11

Notorynchus cepedianus		4	77 ± 8.1		56 ± 5.2	8	2
Dentiraja cerva	11	7	65 ± 4.5	31 ± 8.2		11	7
Heterodontus portusjacksoni	5	0	55 ± 1.4		50 ± 1.5	2	3
Aptychotrema vincentiana	0	4	84 ± 4.7	34 ± 1.5		3	1

Results

I collected 68 host microbiomes (21 *T. dumerilii*, 18 *D. cerva*, 12 *S. australis*, eight *N. cepedianus*, five *H. portusjacksoni*, four *A. vincentiana*) and 14 water column microbiomes resulting in 87,495,310 annotated reads. Microbiomes ranged from 15,435 reads to 4,217,425 reads (median 447,443.5). The microbiome assembly produced 57 metagenome assembled genomes representing seven unique bacterial clades.

Taxonomic Community

Pseudomonadota was the most abundant phylum in all host microbiomes, accounting for up to 90% of the known Elasmobranch microbiome. Water column microbiomes had a lower average abundance of Pseudomonadota (48%). Bacteroidota (3-12%) and Planctomycetota (1-6%) were the remaining phyla with greater than 1% abundance across hosts. Water samples had a high relative abundance of Bacteroidota (19%), Planctomycetota (13%) and Cyanobacteria (5%). Families abundant in shark metagenomes include *Pseudoalteromonadaceae* (0.3-13%), Pseudomonadota unknown (16-17%), Gammaproteobacteria unknown (11-15%), Alphaproteobacteria unknown (6-11%), and *Pseudomonadaceae* (0.1-12%) (Figure 2.1).

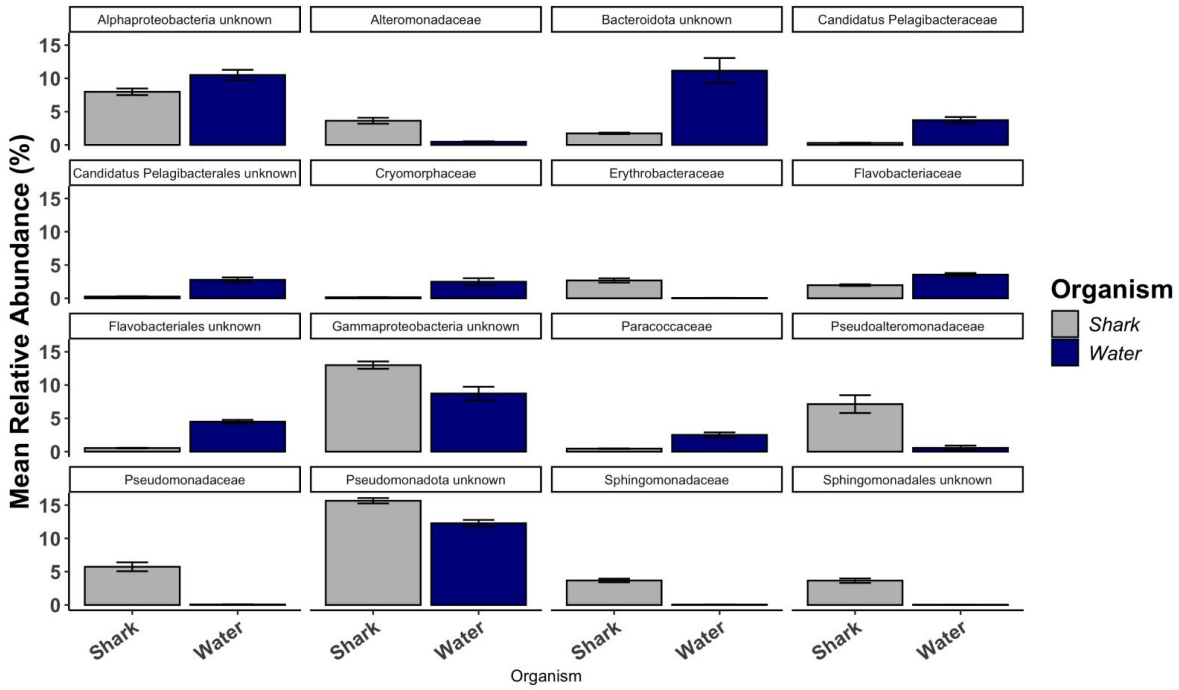

Figure 2.1: Mean relative abundance of the most abundant taxa on Elasmobranch hosts (grey) and the water column (blue).

Host microbiomes were distinct from the water column (PERMANOVA p = 0.001, Pseudo-F = 31.015, df = 1, denominator df = 80) (Supplementary Figure 2.1). PERMANOVA results showed that host and month were significantly different (Host p = 0.038, Pseudo-F =, df = 5, denominator df = 71; Month p = 0.001, Pseudo-F = 16.126, df = 1, denominator df = 71) but the interaction between host and month was not significant (p = 0.144, Pseudo-F 1.3622, df = 3, denominator df = 71). Host microbiomes were not significantly different to each other for any pairwise comparison (Table 2). Within each month (including hosts caught in only one month) host microbiomes were not significantly different (March PERMANOVA p = 0.05, F = 1.897, df = 4; April PERMANOVA p = 0.05, F = 1.746, df = 4). For each of the four host species caught in both months microbiomes were significantly different between March and April (*T. dumerilii* PERMANOVA p = 0.001, F = 13.2, df = 1; *N. cepedianus* PERMANOVA p = 0.03, F =

4.59, df = 1; *D. cerva* PERMANOVA p = 0.002, F = 5.72, df = 1; *S. australis* PERMANOVA p = 0.01, F = 3.47, df = 1). No significant difference in microbiome beta diversity was observed for sex in *S. australis*, *D. cerva*, *N. cepedianus,* or *T. dumerilii*.

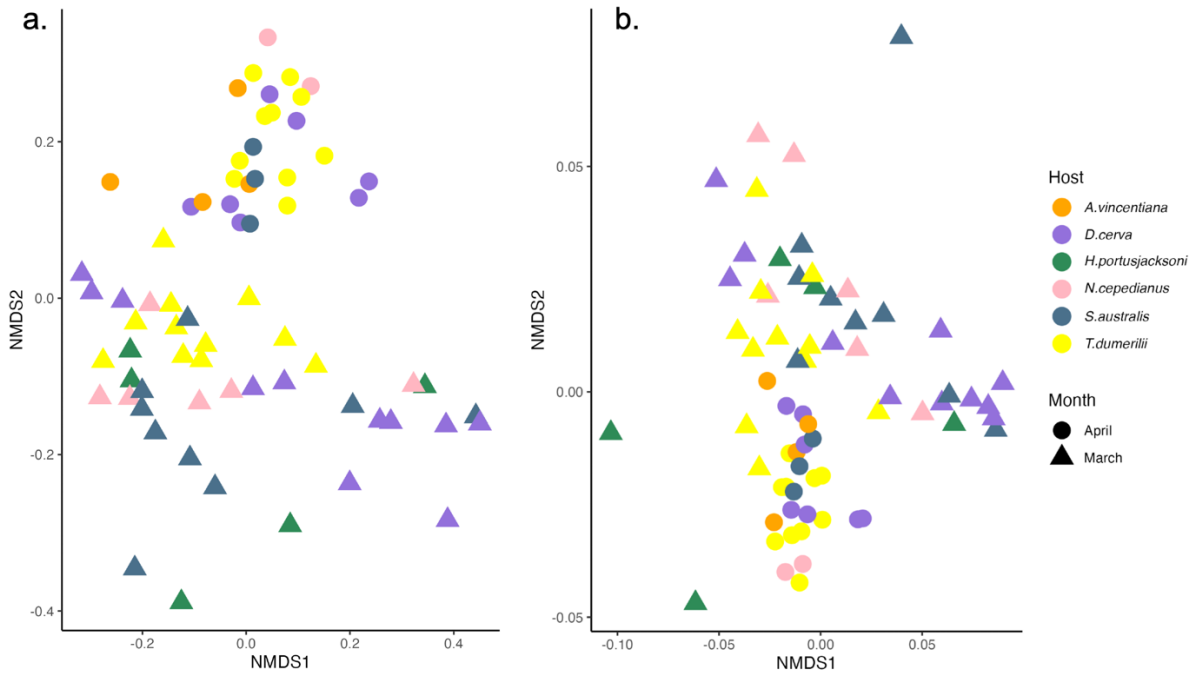

Figure 2.2: NMDS showing the a.) family and b.) Level 3 functional composition of host microbiomes during the two sampling periods. Host microbiomes collected in March have higher dispersion than those collected in April for both taxa and functional composition.

Table 2.2: Pairwise PERMANOVA results for taxonomic composition of host microbiomes. Degrees of freedom is 1 for all tests, Bonferroni method used to calculate p-adjusted

Host Comparison	Sums Of Sqares	F Model	R^2	p-adjusted
T. dumerilii vs *H. portusjacksoni*	0.080	2.302	0.088	0.6
T. dumerilii vs *N. cepedianus*	0.039	1.129	0.040	1
T. dumerilii vs *D. cerva*	0.125	3.308	0.082	0.075
T. dumerilii vs *S. australis*	0.120	3.506	0.102	0.105
T. dumerilii vs *A. vincentiana*	0.038	1.245	0.051	1
H. portusjacksoni vs *N. cepedianus*	0.036	0.727	0.062	1
H. portusjacksoni vs *D. cerva*	0.072	1.498	0.067	1
H. portusjacksoni vs *S. australis*	0.045	1.000	0.063	1
H. portusjacksoni vs *A. vincentiana*	0.094	2.117	0.232	0.69

N. cepedianus vs *D. cerva*	0.062	1.347	0.053	1
N. cepedianus vs *S. australis*	0.028	0.653	0.035	1
N. cepedianus vs *A. vincentiana*	0.052	1.274	0.113	1
D. cerva vs *S. australis*	0.090	2.044	0.068	1
D. cerva vs *A. vincentiana*	0.087	2.004	0.091	1
S. australis vs *A. vincentiana*	0.082	2.139	0.132	0.975

Dispersion did differ significantly between *T. dumerilii* and both *D. cerva* (PERMDISP = 0.009, F = 3.29, df = 1), and *S. australis* (PERMDISP p = 0.003, F = 3.51, df = 1), but not between other species pairwise comparisons. Dispersion for all hosts combined was significantly higher in March than in April (PERMDISP p = 0.001, perms = 999, F = 21.94, df = 1).

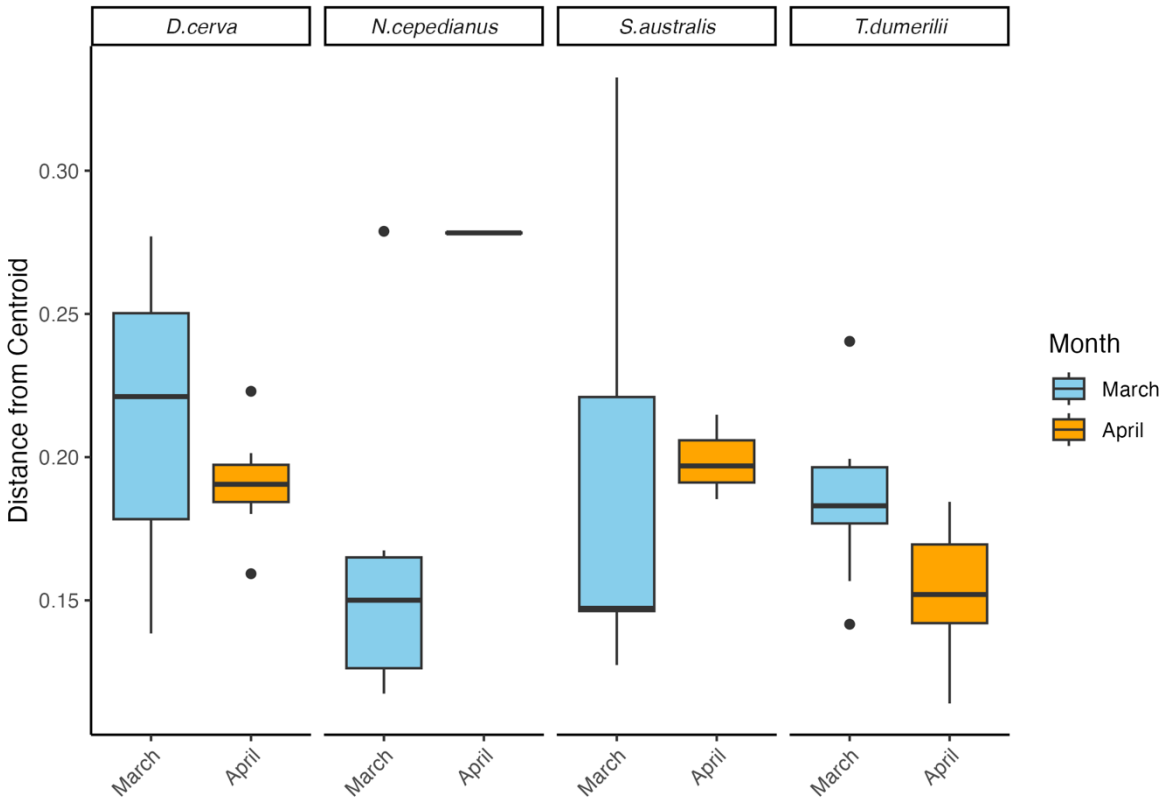

Figure 2.3: Dispersion (distance from centroid) was higher in March (blue) than April (orange) in all hosts, which coincided with more variable environmental conditions.

Alpha diversity (Hill diversity) did not differ between hosts (Kruskal-Wallis chi-squared = 4.49, df = 3, p-value = 0.213) or month (Kruskal-Wallis chi-squared = 0.94, df = 1, p-value = 0.331). Alpha diversity was not different between male and female hosts for *T. dumerilii* (Kruskal-Wallis chi-squared = 0.005, df = 1, p-value = 0.944 or *D. cerva* (Kruskal-Wallis chi-squared = 0.018, df = 1, p-value = 0.892).

DSeq2 showed minimal differentially abundant taxa between pairs of host species ($\alpha = 0.05$). *Trygonorrhina dumerilii* had 68 and 52 differentially abundant families compared to *D. cerva* and *S. australis* respectively. All other host comparisons had fewer than 50 significantly different taxa, some even having zero families with significantly different abundances. Between March and April 414 microbial taxa were differentially abundant ($p < 0.05$) across all hosts. 111 of those taxa had a log fold change of 2 or greater. Six families (*Alcanivoracaceae*, *Cyclobacteriaceae*, *Erythrobacteraceae*, *Marinobacteraceae*, *Pseudoalteromonadaceae*, and Sphingomonadales unknown) were differentially abundant in March and April and had an average relative abundance of at least 1%. Across all hosts only Pseudoalteromonadaceae were more abundant in March than in April (Figure 2.4).

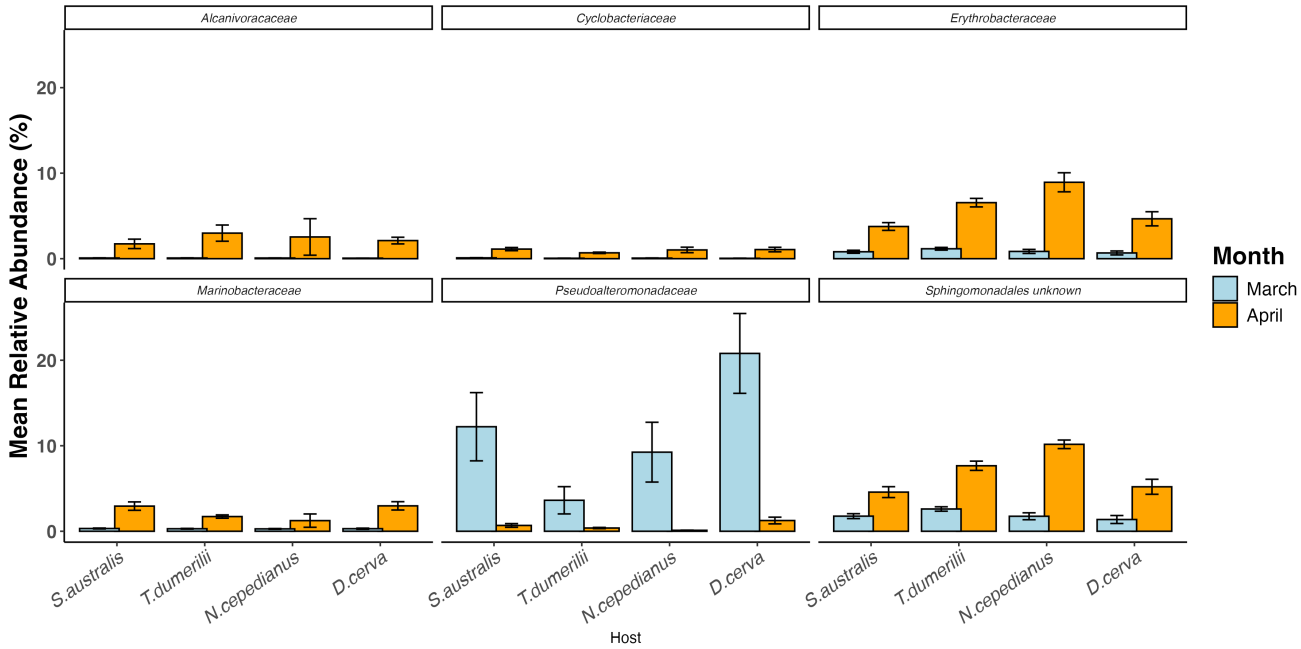

Figure 2.4: Mean relative abundance of microbial families that were differentially abundant in March and April ($p < 0.01$) and had a log fold change of 2 or greater. Only families with at least 1% abundance are displayed. Error bars indicate standard error. Similar trends in abundance occur across all hosts for each bacterial taxa.

Functional Potential

Host microbial Level 1 functions were significantly different to the water column (PERMANOVA p = 0.001, F = 129.84, df = 1). Host Level 1 functions were not significantly different between hosts (PERMANOVA p = 0.187, F = 1.41, df = 5). Overall, Level 1 functions were different between March and April (PERMANOVA p = 0.022, F = 4.76, df = 1). When tested pairwise, only *T. dumerilii* Level 1 functions were significantly different between March and April (PERMANOVA p = 0.002, F = 11.8, df = 1). Level 1 functions in *S. australis* (PERMANOVA p = 0.392, F = 0.962, df = 1), *D. cerva* (PERMANOVA p = 0.646, F = 0.277, df = 1), and *N. cepedianus* (PERMANOVA p = 0.238, F = 1.924, df = 1) microbiomes were stable across sampling dates.

Host Level 3 functions were significantly different across hosts (PERMANOVA p = 0.01, F = 1.91, df = 5), but no individual pair of species was different (Table 2). Dispersion was significantly different between hosts (PERMANOVA p = 0.004, F = 4.5, df = 5). Level 3 functions were significantly different in March and April for all hosts (*T. dumerilii* PERMANOVA p = 0.001, df = 1, F = 10.38, *S. australis* p = 0.03, df = 1, F= 2.49, *N. cepedianus* p = 0.03, df = 1, F = 3.49, *D. cerva* p = 0.02, df = 1, F = 4.66).

Deseq2 analysis showed 385 differentially expressed functions between March and April (greater than 2 lfc and p < 0.01). Of these, 18 differentially expressed functions between March and April had a relative abundance of 0.1% (Figure 2.5). Pathways abundant in March include Septum-associated cell division protein *DamX*, DNA helicase IV, and c-di-GMP phosphodiesterase (EC 3.1.4.52). In April, Wax ester synthase/acyl-CoA:diacylglycerol acyltransferase, Cell surface glycan-binding lipoprotein, utilization system for glycans and polysaccharides (PUL), *SusD* family, and internalin, putative were significantly more abundant than in March (Figure 2.5).

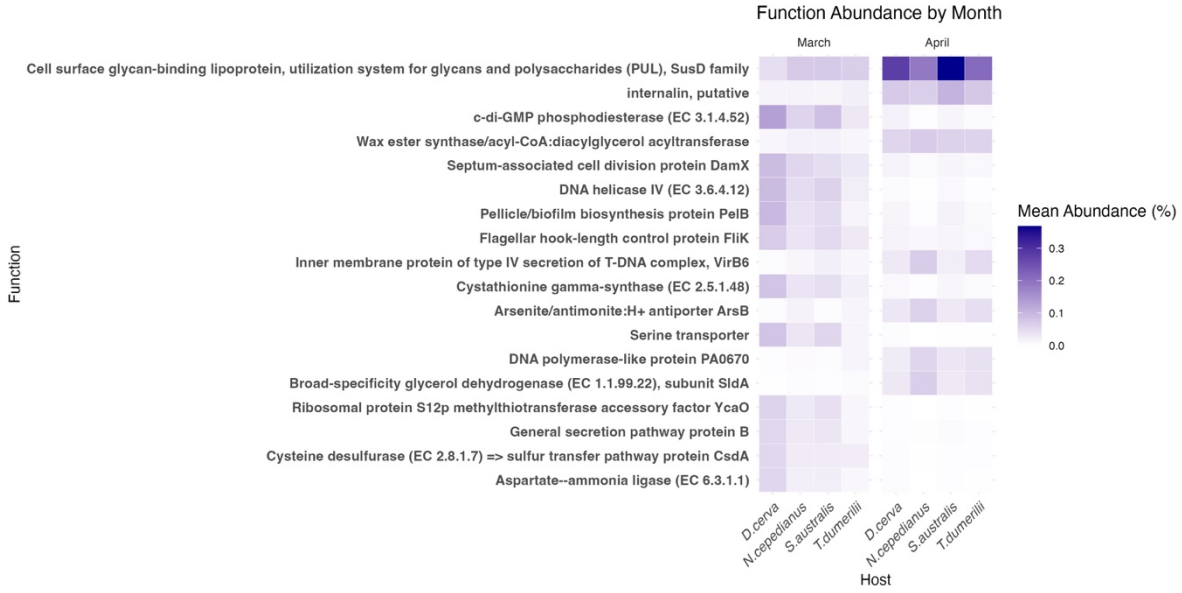

Figure 2.5: Mean relative abundance of Level 3 functional genes for each host in March and April. Functions displayed are significantly differentially abundant (p-adj <0.01 and lfc >2).

Bacterial Abundance

Bacterial abundance was higher in April than March for all hosts and the water column (Figure 2.6). Only fiddler rays had a significantly higher bacterial abundance in April (Kruskal-Wallis chi-squared = 8.7585, df = 1, p-value = 0.003082). Bacterial abundance was generally lower in sharks compared to the water column, though the sample size did not allow for significance testing.

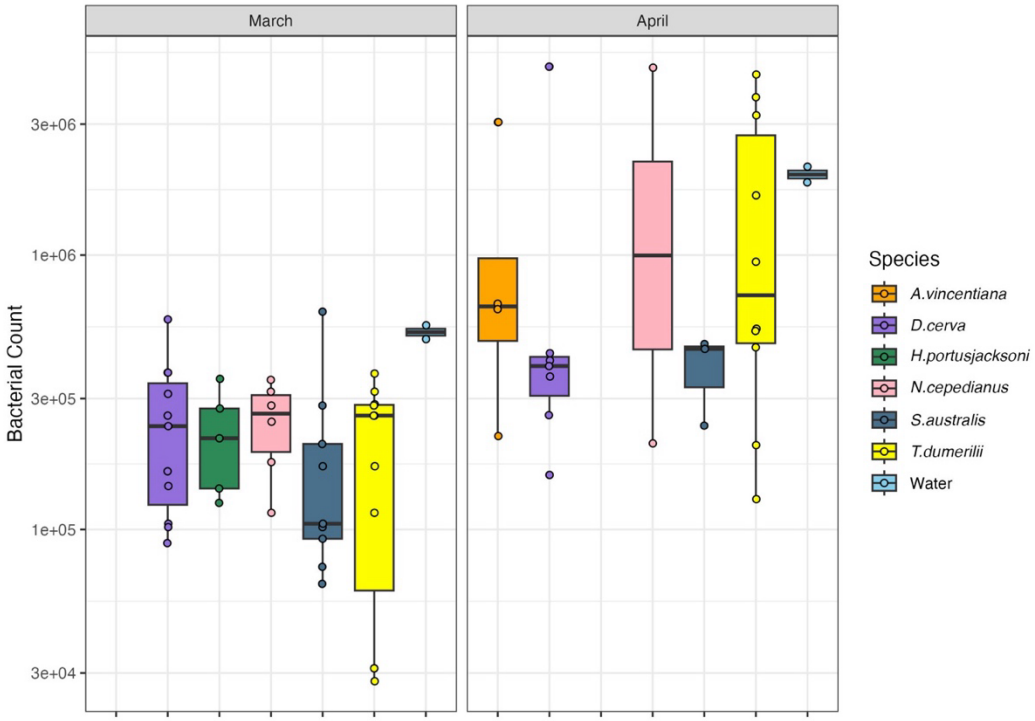

Figure 2.6: Bacterial abundance for each host and water column in March and April.

Metagenome Assembled Genomes

We assembled 57 high quality MAGs with > 70% completeness and less than 10% contamination. MAGs belonged to kingdom Bacteria (15), class Alphaproteobacteria (2), Gammaproteobacteria (3), Betaproteobacteria (10), order Sphingomonadales (21), family *Moraxellaceae* (3), and *Rhodobacteraceae* (3) (Figure 2.7). MAGs within a microbial taxonomic group had similar functional gene profiles and clustered together following phylogeny (Figure 2.8).

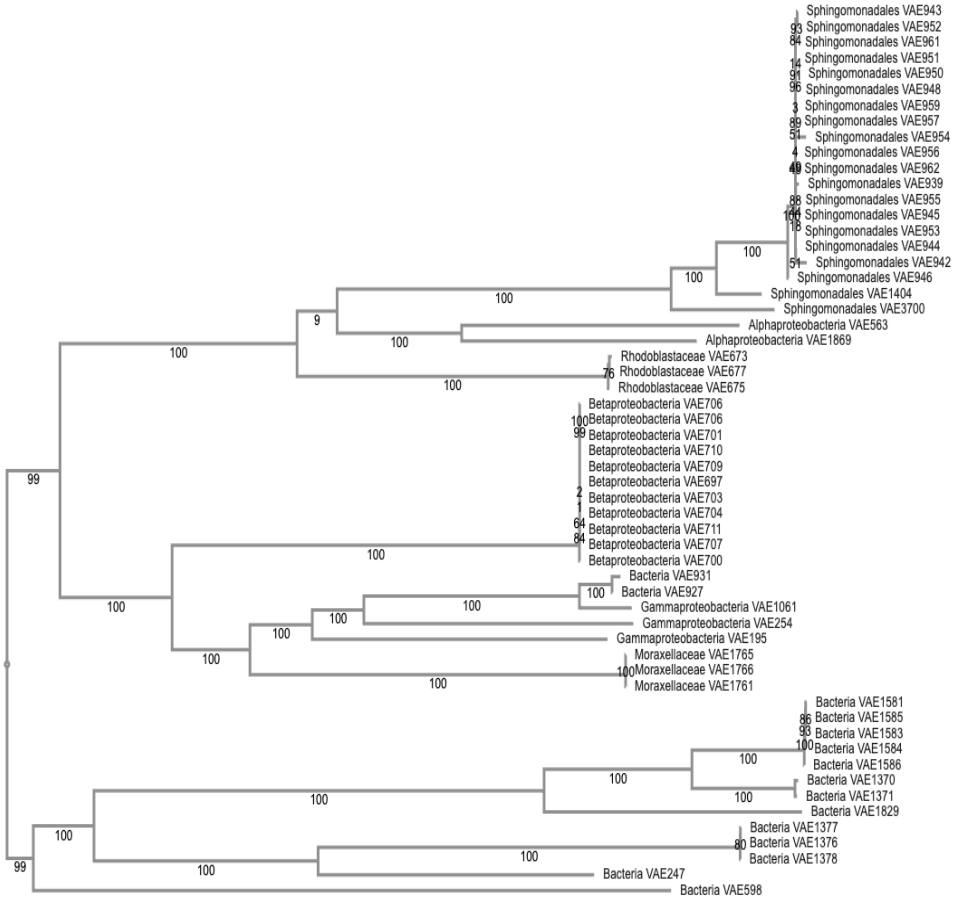

Figure 2.7: Phylogenetic tree of Metagenome Assembled Genomes. Several unique clades of unknown bacteria species are present, evidence of novel microbes.

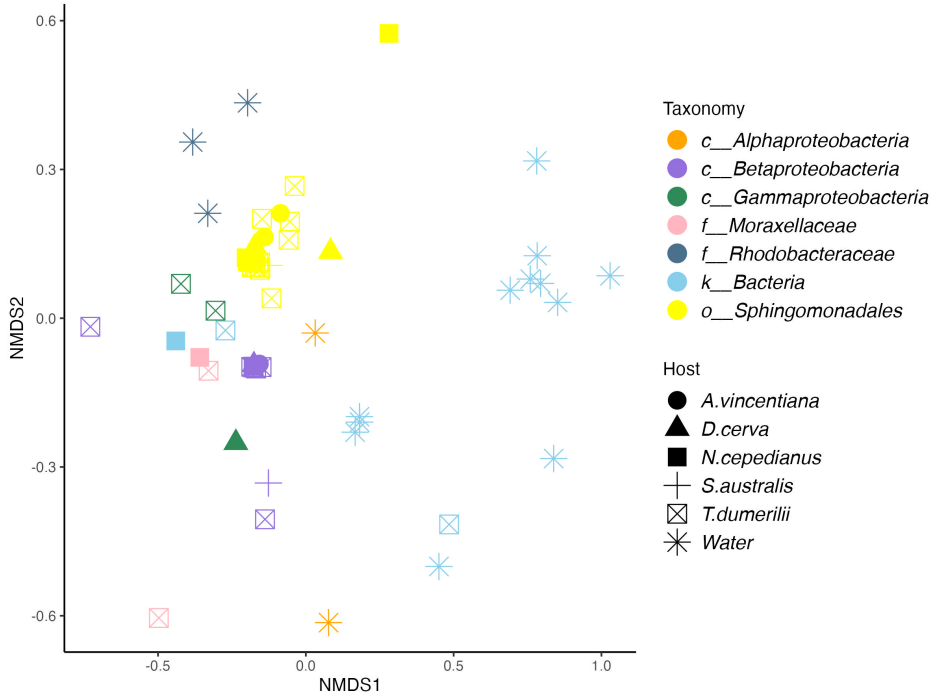

Figure 2.8: NMDS showing metagenome assembled genomes by similarity (Bray-Curtis distance) of subsystem level microbial functions. Functional similarity is clustered by bacterial taxonomy rather than host in which the MAG was derived.

Discussion

Juvenile Elasmobranch microbiomes showed high flexibility with environmental conditions and had not developed into the typical species-specific adult microbiome previously observed in other Elasmobranchs (Clavere-Graciette et al. 2023; Goodman et al., 2024; Hesse et al., 2022). From all eight species of Elasmobranch, the microbiomes of the hosts were different from the water column microbes over the two months, but the microbiomes of each host species were not different from one another. The variation in microbiomes between March and April included taxonomic and functional composition, dispersion and abundance. The bacterial abundance was higher in April than in March in host microbiomes and the water column coinciding with a decrease in temperature and

chlorophyll a, suggesting these are cold adapted microbes. Therefore, juvenile Elasmobranch epidermal microbial communities are influenced by seasonal changes in the environment, both taxonomically and functionally while still maintaining distinct microbiomes from the surrounding water column.

Elasmobranchs typically maintain distinct microbial communities from the water and sediment (Clavere-Graciette et al., 2022; Gonçalves e Silva et al., 2020; Goodman et al., 2023; Kerr et al., 2023) and we identified in the same trend in all eight host species from South Australia (Figure 2.1). However, host microbiomes had similar taxonomic (family) and functional (Level 3) composition within each month (Figure 2.2), which is unusual for Elasmobranch microbiomes which typically vary between hosts, even when sampled in the same environment (Clavere-Graciette et al., 2022; Kerr et al., 2023). The high degree of similarity across host species could be due to host age and the highly flexible microbiome may be a strategy for Elasmobranch host to adapt to the rapidly changing environment that they encounter in shallow waters. The individuals in this study were juveniles, measuring below length at maturity (Ebert 2002; Ellis et al., 2021; Izzo and Gillanders 2008). Microbiome shifts with ontogeny occur in several marine species, including, little skate (*Leucoraja erinacea*) (Mika et al., 2021), brown trout (*Salmo trutta*) (Streb et al., 2025), and seabass (Rosado et al., 2021). Little skate (*Leucoraja erinacea*) skin and gill microbiomes were specific at each stage of development; embryonic skin was distinct from adult skin, and it is hypothesized that denticle development affected epidermal communities (Mika et al., 2021). Denticle morphology and distribution vary with shark age (Vaz et al., 2023), which may cause microbiomes to be distinct in adult Elasmobranchs. Dermal denticle topography and mucus composition are linked to epidermal microbial communities (Goodman et al.,

2024; Kerr et al., in review), and we suggest that these skin-microbiome relationships may not be developed yet in juveniles. Though hosts are not distinct from one another they maintain selectivity from the water column. Skate embryos maintain distinct microbial communities from the water (Mika et al., 2021). Elasmobranchs appear to have an innate selective mechanism driving distinction from the environment even as juveniles, while adaptive changes (like denticles and mucus) are acquired during development driving differences between species in adulthood. The continued distinction of Elasmobranch skin microbiomes, both demersal and pelagic, from the water column bacterioplankton demonstrates the selective nature of both denticle dominated (pelagic and demersal sharks) and mucus dominated (rays and skates) skin types in juveniles and adults. Distinction of host microbiomes from the surrounding water microbiome suggest host microbiomes are not recruiting bacteria from the water, rather they are affected by other environmental factors.

Ontogenetic shifts in microbiome composition may coincide with changes in diet, habitat use, or other physiological changes (Price et al., 2017). In green sea turtles, cloaca microbiomes shift when turtles transition from herbivorous diets to omnivorous diets during development (Price et al., 2017). In catfish (*Ictalurus punctatus*), gut microbiomes stabilize as individuals age (Burgos et al., 2018). *Notorynchus cepedianus* and *H. portusjacksoni* change diets during development (Ebert 2002; Powter et al., 2010). In some coral reef fishes, juvenile gill microbiomes are less influenced by host diet than adults, suggesting host specific factors play a larger role in shaping the gill microbiome of adult fish (Pratte et al., 2018). Microbiome composition did not vary between males and females of any coral reef fish (Pratte et al 2018) or other fish species that lack sexual dimorphism, like tuna and salmon (Gadoin et al., 2023; Uren Webster et al., 2018). Similarly in sharks, male and

female black tip reef shark skin microbiome composition is similar (Pogoreutz et al., 2017). Given that hosts in this study have not reached maturity, it is not surprising that microbiomes are similar in females and males.

Skin microbiomes of each host species (*T. dumerilii*, *S. australis*, *D. cerva*, and *N. cepedianus*) was significantly different between March and April (Figure 2.2). Location (ie. environment) plays a role in shaping skin microbial communities in black tip reef sharks, round rays, and whale sharks (Doane et al., 2023; Kerr et al., 2023; Pogoreutz et al., 2019). Environmental impacts on ray microbiomes are particularly pronounced between wild and captive rays (Clavere-Graciette et al., 2022; Pinnell et al., 2021) but not wild and captive sharks (Goodman et al., 2022). Epidermal microbial communities of leopard sharks differed in taxonomic composition over a period of four summers, but functions were maintained (Doane et al., 2022) suggesting seasonal shifts in the water column do not affect microbiome functions in adult sharks. In this study, skin microbiomes changed across two sampling events, indicating seasonal environmental influence on juveniles. Dispersion was higher in March for both taxa and functions compared to April (Figure 2.3). Increased habitat disturbance increases microbiome dispersion and stochasticity (Neely et al., 2022). On the Bonnie Coast adjacent to the Gulf St. Vincent, upwelling occurs from November to April, resulting in warmer sea surface temperature and higher chlorophyll concentration in the gulf water column during these periods (Kampf 2025). In March the concentration of chlorophyll a was higher and more variable (1.37 ± 1.2 mg/ m^3) than in April (1.27 ± 0.89 mg/m^3) (IMOS 2025). Sea surface temperature was also higher and more variable in March (21.63 ± 0.82 °C) than in April (19.39 ± 0.52 °C) (IMOS 2025). The abundance of bacteria was lower for all samples at the higher temperatures in March (Figure 2.6). Meta-analysis of

terrestrial and aquatic host microbiomes showed that temperature had a significant effect on microbiome composition (Li et al., 2023). An increase in microbiome dispersion in response to environmental fluctuations, particularly warming, indirectly affect vertebrate fitness due to microbiome dysbiosis (Greenspan et al., 2020). Here both taxonomy and functional gene composition were more dispersed in March during the warmer weather and less variable in April. Juvenile Elasmobranchs may be more susceptible to adverse changes in the environment due to climate change and other anthropogenic disturbances and flexible microbiomes may help temper these changes. Future studies should include temperature at depth and other abiotic environmental data to determine the influence of abiotic factors on host microbiomes.

Host microbiomes had a high average relative abundance of *Pseudoalteromonadaceae*, Alphaproteobacteria unknown, Gammaproteobacteria unknown, *Pseudomonadaceae*, and *Sphingomonadaceae*. *Pseudomonadaceae* is a bacterial family commonly found in shark epidermal microbial communities (Doane et al., 2022, Goodman et al., 2022). *Pseudomonadaceae* decreased in captivity compared to wild leopard sharks, but not by a significant amount (Goodman et al., 2022). While *Pseudomonadaceae* are common in shark microbiomes the relative abundance fluctuates, suggesting that it may be more impacted by environmental factors or shifts in other members of the community rather than static host factors like skin structure (Doane et al., 2022). Sphingomonadales are also common, but less abundant members of shark skin microbiomes (5-10%) (Doane et al., 2023; Goodman et al., 2023). Sphingomonadales are aerobic, rod-shaped, and are unique because of the glycosphingolipids in their cell membranes (Balkwill et al., 2006). An

unknown Sphingomonadales clade had different relative abundance in each host in March and April, but the relative abundance remains within the range observed in shark microbiomes previously (Doane et al., 2022; Goodman et al., 2022). Many Sphingomonadales MAGs were recovered from multiple hosts species indicating their prevalence in Elasmobranch microbiomes (Figure 2.8). Sphingomonadales have diverse metabolic range and ability to degrade complex molecules from the environment (Balkwill et al., 2006) which may increase their ability to persist in the microbiome during environmental fluctuations. Sphingolipids increased the microbial growth of trout skin microbes and reduced host expression of sphingolipid genes (Sepahi et al., 2016). The increase in Sphingomonadales bacteria in April could be providing sphingolipids to other members of the microbial community increasing growth and therefore increased bacterial populations (Figure 2.6), though further experiments would be required to confirm the role of Sphingomonadales in the microbiome.

Microbial functional potential varied between sampling dates (Figure 2.2b) and the abundance of specific gene pathways fluctuated similarly across host species (Figure 2.5). In March genes involved in cell signalling and biofilm formation were significantly more abundant, particularly c-di-GMP phosphodiesterase (EC 3.1.4.52) and pellicle/biofilm biosynthesis protein *PelB*. Both genes are crucial for quorum sensing (Diaz et al., 2021), which could affect microbe-microbe interactions within the microbiome during assembly (Su et al., 2023). In April the most abundant gene pathway was the *SusD* family (Figure 2.5). The Sus operon is important for bacterial recognition, binding and transport of glycans (Brown & Koropatkin 2021, Foley et al., 2016, Tuson et al., 2018). *SusD* is involved in glycan binding, which is integral in host microbiome interactions in mucus (Van tassell et

al., 2011). Elasmobranchs mucus contains a diversity of glycans (Bachar-Wikstrom et al 2023a) and microbes have the genetic capability to metabolise Elasmobranch glycans (Kerr et al., in review). The abundance of *SusD* (glycan binding gene) in April indicates bacteria are closely interacting with host mucus (Kerr et al., in review).

Trawling is known to stress Elasmobranchs, even if that stress does not result in immediate mortality (Mandelman & Farrington 2007). While there could be a homogenising effect of trawling on microbial communities, between one and five animals were sampled from each of the 24 trawls collected March and 48 trawls collected in April across two vessels, and an individual trawl effect was not observed. Skin microbiomes of Elasmobranchs collected during trawling operations were distinct across co-collected species (Kerr et al., 2023; Lyons et al., 2024). While trawling might have some effect, catch method is not the driving factor of microbiome similarity of hosts within each month.

From the metagenome assemblies, 57 MAGs were recovered representing seven bacterial clades (Figure 2.7). Family was the most resolved taxonomic level for recovered MAGs. A total of 15 MAGs could only be identified as Bacteria, evidence of novel bacterial genomes in marine microbial communities (Figure 2.8). Previous metagenomic studies on Elasmobranchs has demonstrated a significant challenge in annotation rates (Goodman et al., 2024; Hesse et al., 2022; Kerr et al., 2023). MAGs provide a reference and culture independent approach to identifying novelty in microbial communities (Papudeshi et al., 2017). MAGs VAE931 and VAE927 are both novel *Alcanivorax* species. *Alcanivorax* were a persistent member of the *T. semifasciata* leopard shark skin microbiome over time, though they fluctuated in relative abundance. *Alcanivorax* were correlated with ammonia assimilation, which is hypothesised to benefit other member of the *T. semifasciata* skin

microbiome (Doane et al., 2022). MAG 247 is most closely related to the genus *Gimesia*, which falls within Planctomycetota, a phylum characterized by a high diversity of Carbohydrate Active Enzymes (CAZymes) (Klimek et al., 2024). Host mucus carbohydrates could be a source of energy for symbiotic bacteria, leading to a close host-microbiome relationship (Kerr et al., in review).

Here I demonstrate that co-occurring juvenile Elasmobranchs share similar taxonomic and functional epidermal microbial communities to each other when sampled in the same location. I also observe a shift in microbial communities (taxa and functions) between two sampling periods (30 days apart). We suggest changes in the environment have a strong influence on juvenile Elasmobranch microbiomes, including composition and microbial abundance. We also recovered 15 novel Metagenome Assembled Genomes highlighting the novelty of Elasmobranch microbiomes.

CHAPTER 3: STINGRAY MICROBIOMES ARE SPECIES SPECIFIC WITH LOCAL ADAPTATIONS

Context

Sharks and rays are closely related, but have distinct ecology, life history, and skin morphology. Sharks have distinct dermal denticle structure, but in rays dermal denticles are reduced or absent. Sharks have species specific skin microbiomes and low variation in microbial communities among individuals of the same species, hypothesized to be a result of denticles structuring microbial communities. In this chapter I aimed to identify patterns of host specificity in two stingray species with increased mucus coverage and reduced denticles. I also compare the microbiomes across two locations to identify environmental influence on stingray skin microbiomes. This is the first investigation of wild stingray microbiome taxonomy and functional potential with shotgun metagenomics. This work is published in Frontiers Microbiology: Women in Aquatic Microbiology special edition.

Abstract

Marine host-associated microbiomes are affected by a combination of species-specific (e.g., host ancestry, genotype) and habitat-specific features (e.g., environmental physiochemistry and microbial biogeography). The stingray epidermis provides a gradient of characteristics from high dermal denticles coverage with low mucus to reduce dermal denticles and high levels of mucus. Here we investigate the effects of host phylogeny and habitat by comparing the epidermal microbiomes of *Myliobatis californica* (bat rays) with a mucus rich epidermis, and *Urobatis halleri* (round rays) with a mucus reduced epidermis from two locations, Los Angeles and San Diego, California (a 150 km distance). We found that host microbiomes are species-specific and distinct from the water column, however

composition of *M. californica* microbiomes showed more variability between individuals compared to *U. halleri*. The variability in the microbiome of *M. californica* caused the microbial taxa to be similar across locations, while *U. halleri* microbiomes were distinct across locations. Despite taxonomic differences, Shannon diversity is the same across the two locations in *U. halleri* microbiomes suggesting the taxonomic composition are locally adapted, but diversity is maintained by the host. *Myliobatis californica* and *U. halleri* microbiomes maintain functional similarity across Los Angeles and San Diego and each ray showed several unique functional genes. *Myliobatis californica* has a greater relative abundance of RNA Polymerase III-like genes in the microbiome than *U. halleri*, suggesting specific adaptations to a heavy mucus environment. Construction of Metagenome Assembled Genomes (MAGs) identified novel microbial species within *Rhodobacteraceae*, *Moraxellaceae*, *Caulobacteraceae*, *Alcanivoracaceae* and Gammaproteobacteria. All MAGs had a high abundance of active RNA processing genes, heavy metal, and antibiotic-resistant genes, suggesting the stingray mucus supports high microbial growth rates, which may drive high levels of competition within the microbiomes increasing the antimicrobial properties of the microbes.

Introduction

Host-associated microbiomes directly affect host health and development (Apprill, 2017; Cullen et al., 2020; Llewellyn et al., 2014; Malard et al., 2021). Microbiomes are host specific, vary with extrinsic factors, such as temperature and location and are impacted by climate change (Lima et al., 2020, 2022; Wilkins et al., 2019). Elasmobranchs, which includes sharks, rays, and skates, regulate the health of oceanic ecosystems (Sandin et al., 2008), but the connection between microbes and Elasmobranch health is challenging to resolve. To date, Elasmobranch-microbe relationships remain poorly understood, with the

microbiomes of 37 shark and only 6 ray species out of 1,300 species being investigated (Perry et al., 2021).

Batoidea diverged from sharks between 200 and 229 million years ago, branching into modern rays about 140 million years ago (Aschliman et al., 2012). Batoidea includes over 600 species, about half of the diversity within Chondrichthyes (Aschliman et al., 2012; Kousteni et al., 2021). Twenty-two species of ray live along the coast of California accounting for over a quarter of Elasmobranch diversity in the region (Ebert, 2003). In California, rays including *Myliobatis californica* (bat rays), *Urobatis halleri* (round rays), are meso-predators, feeding on small invertebrates, and serving as prey for larger Elasmobranchs and marine mammals (Gray et al., 1997; Last et al., 2016). Rays disturb sediment to uncover prey, creating feeding pits which have a significant impact on benthic infauna communities by exposing otherwise sequestered resources and creating habitat for other organisms (van Blaricom, 1982). Sharks and rays regulate oceanic food webs and contribute to tourism economics (Healy et al., 2020; Newsome et al., 2004). Rays are key members of coastal ecosystems across the globe including sand flats, kelp forests, seagrass meadows, and coral reefs (Gray et al., 1997; Lyons et al., 2014; O'Shea et al., 2012). Elasmobranchs have long lifespans and late maturity which make them vulnerable to overexploitation, and many of these species are threatened globally (Domingues et al., 2018; Johri et al., 2019, 2020; Kousteni et al., 2021). Microbiome exploration in Elasmobranchs using whole genome (shotgun) sequencing has allowed reconstruction of host genomes, which aids in resolving phylogenies, and can support conservation efforts (Doane et al., 2018; Johri et al., 2019). Elasmobranch microbiomes remain an important ecosystem of discovery, which has been focused on sharks while rays remain understudied (Clavere-

Graciette et al., 2022; Gonçalves e Silva et al., 2020; Kearns et al., 2017; Pinnell et al., 2021).

The epidermis of Chondrichthyes is covered in dermal denticles, which are tooth-like placoid scales. Stingrays, unlike most sharks, have a thick layer of mucus with a reduced covering of dermal denticles. Both the dermal denticles and mucus act as the first defence against injury and invading pathogens (Meyer & Seegers, 2012), but where denticles are sparse, epidermal mucus serves as a barrier between the host and the environment. Proteases and antimicrobial peptides are present in ray mucus and reduce the survival of harmful microbes (Vennila et al., 2011). Stingray mucus, and the microbes within, produce antimicrobial molecules preventing infections of wounds resulting from feeding and mating (Conceição et al., 2012; Kajiura et al., 2000; Pogoreutz et al., 2019; Ritchie et al., 2017). The stingray epidermis, (and its unique mucus properties) serves as an interesting model system to compare with the microbiomes of sharks, which are covered in dermal denticles (Doane et al., 2022; Ritchie et al., 2017). Sharks with a dense denticle structure have microbiomes that are highly similar across individuals, species specificity, and show phylosymbiosis (Doane et al., 2017, 2020, 2022). The epidermis of teleost fishes is covered by a layer of thick mucus, similar to the mucus found on stingrays (Meyer & Seegers, 2012) and the microbiome of teleost fish is species-specific, but epidermal microbiomes generally have low similarity across individuals of the same species (Chiarello et al., 2018). Therefore, we predict that the mucus associated with rays will influence the taxonomy of the microbiome, but the functions of the ray microbiome will be similar to that of sharks, since they share similar metabolic characteristics, such as high levels of osmolytes (urea and

TMAO (Trimethylamine N-oxide)) in the blood, and bioaccumulation of heavy metals (Withers et al., 1994a,b).

Stingray epidermal microbiomes vary between wild and captive individuals of the same species (Clavere-Graciette et al., 2022; Pinnell et al., 2021). The epidermal microbial community of cow-nose rays (*Rhinoptera bonasus*) from an aquarium, had lower diversity compared with of the surrounding environment suggesting the ray skin microbiome is selective (Kearns et al., 2017). *Hypanus americanus* (southern stingray) microbiomes are more similar to shark microbiomes than water column communities (Caballero et al., 2020). Yellow stingrays (*Urobatis jamaicensis*) microbiomes were distinctive across wild, aquarium-housed and aquarium-born rays. The wild caught rays had a lower abundance of Bacteroidetes, an abundant pelagic microbe, compared with those that were aquarium born, which suggests the filtered aquarium water environment has an impact on the skin microbiome (Pinnell et al., 2021). Leopard sharks (*Triakis semifasciata*) skin microbiomes did not show differences across captive and wild individuals, suggesting that skin properties could be contributing to microbiome stability (Goodman et al., 2022). There is evidence to suggest that captive status plays a major role in structuring Elasmobranch mucus microbiomes, the driving force of these shifts in microbial communities is unknown. Therefore, we compare the effect of species-specific and habitat-specific drivers on the structure of the ray skin microbiomes in the wild.

We use whole genome (shotgun) sequencing metagenomics, which unlike 16S amplicon sequencing uses no primers and has allowed reconstruction of microbial genomes (Setubal, 2021). All ray microbiomes research has been conducting with amplicon sequencing (Clavere-Graciette et al., 2022; Gonçalves e Silva et al., 2020; Kearns et al.,

2017; Pinnell et al., 2021), leaving novel microbes and functions of ray microbiomes understudied. Shotgun metagenomics requires higher sequence depth than 16S sequencing, but allows assembly of the sequences together to construct Metagenomic Assembled Genomes (MAGs), which are near complete microbial genomes (Papudeshi et al., 2017; Tully et al., 2018). This process allows the identification of novel microbes that cannot be identified by 16S alone. Using reference independent assemblers avoids database bias, one of the limitations of shotgun metagenomics (Quince et al., 2017).

We used shotgun metagenomics to describe the epidermal microbiome two species of wild Myliobatiforms, *Myliobatis californica*, and *Urobatis halleri* at two locations along the California coast. These rays have varying skin characteristics *Myliobatis californica* has reduced dermal denticles and high mucus production (Figure 3.1) and *Urobatis halleri* has less mucus and a higher covering of dermal denticles, which we hypothesize will be reflected in the characteristics in the skin microbiome. Our work contributes to filling the knowledge gaps on stingray microbiomes by identifying that stingray microbiome are species-specific. In *U. halleri*, location affected the microbiomes, but this effect was lost in the *M. californica* that had high mucus production.

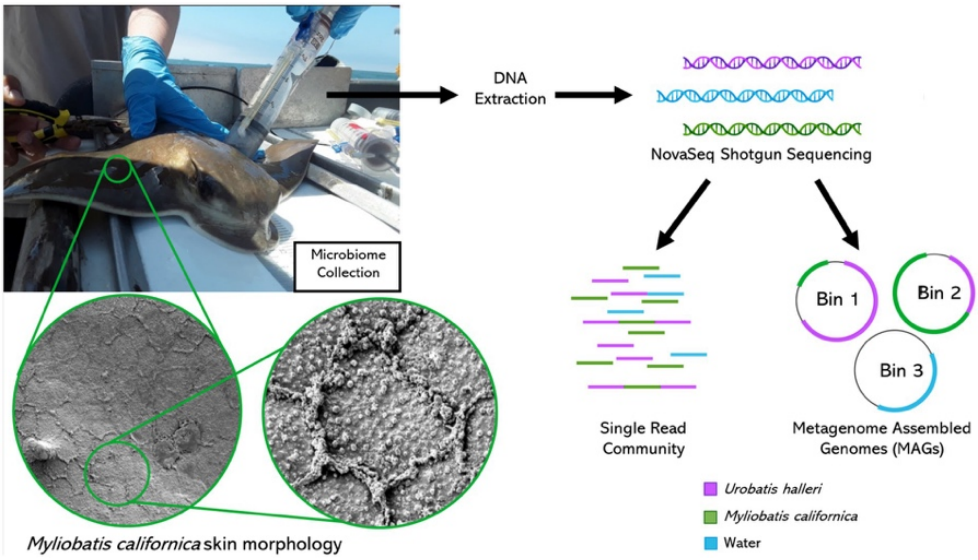

Figure 3.1. Metagenomics sample collection, processing, and bioinformatics analysis. *Myliobatis californica* skin scanning electron microscope images left: 1400X right: 6500X magnification.

Methods

Microbiome samples were collected from individuals along the California coast between Los Angeles Harbor and San Diego Bay. Sampling was conducted opportunistically during California Department of Fish and Wildlife halibut trawls in April and October of 2019. A small trawl was deployed for 10 min at a time at each site, with 5 deeper trawls (about 20 m) and 5 shallower (about 8 m). Elasmobranchs were retrieved and placed into containers with fresh seawater and sorted by species. Depending on location, zero to two Elasmobranchs were collected per trawl, thus multiple trawls were required to obtain replicate microbial samples from each site and species. Microbiome samples were collected using a blunt ended two-way syringe (50 mL) called a "supersucker" (Figure 3.1). Four supersuckers, filled with sterile seawater which was flushed against the skin of the organism and microbial slurry was recollected back into the syringe, via a two-way valve collecting ~200 mL from each organism (Doane et al., 2017, 2020, 2022). The resulting microbial

slurry is filtered through a 0.22 μm sterivex filter to capture microbes. All stingray microbiome samples were collected on the dorsal side of the organism avoiding the spine and providing consistency in sampling location. However, no difference has been observed between the dorsal and ventral sides of R. bonasus (Kearns et al., 2017). Water samples (about 2 liters per sample) were filtered through a 0.22 μm sterivex filter. Sterivex filters were stored on ice until they could be transported to a − 20°C freezer for long term storage. Sampling was conducted in compliance with IACUC guidelines (18–05-007D & 17–11-010D). Once samples were collected, all organisms were returned to the ocean. A total of 15 *M. californica* (Los Angeles n = 6 and San Diego n = 9) and 16 *U. halleri* (Los Angeles n = 8 and San Diego n = 8) had appropriate metagenomes for analysis.

DNA from host associated and water microbiomes were extracted using a modified column purification method with the Nucleospin tissue kit by Macherey-Nagel (Doane et al., 2017). Stingray metagenomes were sequenced at Microbial Genome Sequencing Center on the NovaSeq platform using the Illumina Nextera XT kit. Shotgun libraries of water samples were prepared using Swift 2S Plus Kit and manufacturer protocol (Swift Biosciences) sequenced at SDSU using the Illumina MiSeq platform. Metagenomics was used rather than meta-transcriptomics, as metagenomes describe the functional genes that are important for the microbiome (Coelho et al., 2022; Dinsdale et al., 2008) rather those that are being transcribed at the time of sampling. Sequences can be accessed using the BioProject number PRJNA837707; sample accession numbers range from SRR19392779 to SRR19392812 (Supplementary Table 3.1).

Metagenomes were annotated using a “snakemake” pipeline developed by Edwards (2020). Sequences were checked for quality using Prinseq software and reads with fewer

than 60 base pairs, quality mean below 25 and more than 1 unidentified base were removed, and Poly A and T tails were trimmed by 5 base pairs (Schmieder et al., 2011). FOCUS and SUPERFOCUS was used to determine the taxonomic identity of the sequences and for the identification of functional genes (SEED Subsystem levels 1, 2 and 3) present in the metagenomes (Overbeek et al., 2014; Silva et al., 2014, 2016). To prevent unrelated microbes being grouped together as single "unknown family," unknown reads were manually identified using the next highest positively identified classification (e.g., order, class, phylum). Read abundances were transformed into proportions to allow for analysis between metagenomes, which have variable number of sequences per library, which is preferred over rarefaction (Calle, 2019; Mc Murdie and Holmes, 2014; Quince et al., 2017) and data was transformed using a fourth root transformation (Lima et al., 2020). Unique diversity and Shannon diversity at the family level were compared across locations using Welch Two Sample t-test. Metagenomes were compared using a Bray–Curtis similarity matrix followed by PERMANOVA (Permutational multivariate analysis of variance) and PERMDISP (Permutational analysis of multivariate dispersions) (Anderson, 2017; Anderson et al., 2017) which were used to test for significant differences in microbiome across host species and locations. PERMANOVA takes a permutation approach to identify whether the microbial community is different across variables (host species or location) and SIMPER (similarity of percentages) identifies which microbial taxa or gene function was contributing the differences. PERMDISP calculates a centroid for the group of samples (i.e., all *U. halleri* metagenomes for example) and calculated the distance from the centroid to each of the samples within the group, the larger number the greater the variation between microbiomes. PCO (Principal Component Ordination) was used to visualize relationships between the

microbiomes at family and SEED Level 3 Subsystems. ANOVA (analysis of variance) and Tukey–Kramer test were used to identify significant differences between functional gene potential of ray microbiomes and the water column (Lima et al., 2022). All multivariate statistical tests and diversity indices were conducted using Primer 7 (7.0.17) with PERMANOVA+ (Clarke and Gorley, 2015). All univariate statistics and visualizations were conducted with R using the ggplot2 package (Wickham et al., 2016). While there is constant renaming of microbial groups, for example, Proteobacteria recently being renamed Pseudomonadota phylum (Oren and Garrity, 2021), we have reported the taxonomy as it appears in NCBI.

Metagenome Assembled Genomes (MAGs) were co-assembled using all 34 metagenomes in this study. Reads (about 150 bp) are merged into longer sequences called contigs using Megahit (total 3,870,948 contigs assembled (Li D. et al., 2015). Contigs >1,500 base pairs (102,557 contigs) were binned into 36 bins with 95,155 contigs using Metabat2. Binning uses the characteristics of each contigs to group similar contigs into a 'bin'. These characteristics include, contig coverage, GC content, Kmer frequencies (Kang et al., 2019; Papudeshi et al., 2017). GraphBin was used to refine binning by utilizing the assembly graph connections, increasing the number of contigs included in the 36 bins to 570,096 contigs (Mallawaarachchi et al., 2020). CheckM identified 16 of the refined bins to have >70% completeness using bacterial marker genes (Parks et al., 2015). Five bins contained <10% contamination (Papudeshi et al., 2017; Parks et al., 2015, 2017) and these bins are described to meet high quality bins using the minimum information for metagenomic assembled genomes (Bowers et al., 2017). The five high quality bins were uploaded to PATRIC (Pathosystems Resource Integration Center) where the relative

abundance of SEED level 3 functional genes was transformed by square-root, and a dendrogram heatmap was used to compared across bins (Aziz et al., 2008; Clarke and Davis et al., 2020; Gorley, 2015; Overbeek et al., 2014). The development of MAGs from single read sequences enables the annotation of entire genes and operons, thus providing improved gene descriptions and whereas metagenomes would be annotated with a metagenomic tool, MAGs are annotated with genomic tools, such as PATRIC. PATRIC's similar genome finder identified the most similar reference genome, which may be a MAG, to which each bin was compared using FastANI (Davis et al., 2020; Jain et al., 2018).

A tissue sample was collected using a small 6 mm biopsy punch on the dorsal surface of a captive *M. californica* individual (not included in the microbial analysis in this study). The tissue samples were rapidly frozen in liquid nitrogen. They were removed frozen and then dropped into 2.5% glutaraldehyde in a 0.1 mol cacodylate buffer. They were then fixed overnight at room temperature, washed, and then dehydrated throughout a degraded series of alcohols. The samples were critical point dried with Sam Dri critical point dryer, mounted onto carbon-coated stubs, coated with 6 nm platinum, and observed in Quantas 450 FEG SEM. Three to five high-resolution images (100x magnification) were taken.

Results

Microbial taxonomic composition

Stingray microbiomes (n = 31) yielded 116,346,596 high quality sequences, with an average of 3,753,116 sequences per sample (Supplementary Table 3.1). *M. californica* (n = 15) and *U. halleri* (n = 16) microbiomes were composed of 415 microbial families. Water samples from Los Angeles (n = 1) and San Diego (n = 2) yielded 1,051,965 sequences with an average of 350,655 sequences per sample.

Myliobatis californica microbiomes contained fewer unique families (mean 329.1 ± 48.8) and had lower Shannon diversity (H = 5.6 ± 0.13) than *U. halleri* microbiomes (mean families 398.44 ± 13.8 and H = 5.81 ± 0.04). *U. halleri* and *M. californica* had significantly different evenness ($t = 3.80$ $p < 0.001$). *Myliobatis californica* and *U. halleri* microbiomes showed no significant difference in Shannon diversity between San Diego and Los Angeles locations ($t = -1.737$, $df = 11.651$, value of $p = 0.108$ and $t = -1.602$, $df = 13.89$, value of $p = 0.1316$ respectively) (Figure 3.2). Twelve families were present in host and water microbiomes with a relative abundance of ≥10% in at least one sample, but the relative abundance of these families varied between the rays and water column (Figure 3.3). Eleven of the most abundant families belong to Proteobacteria. Within Proteobacteria, six of the 12 most abundant microbes belong to the Gammaproteobacteria clade. *Alteromonadaceae*, *Pseudoalteromonadaceae*, *Pseudomonadaceae*, *Sphingomonadaceae*, and *Vibrionaceae* are present in greater relative abundance in host microbiomes compared with the water column. A novel *Alteromonadaceae* family was identified in greater relative abundance in ray microbiomes compared to the water column (Figure 3.3).

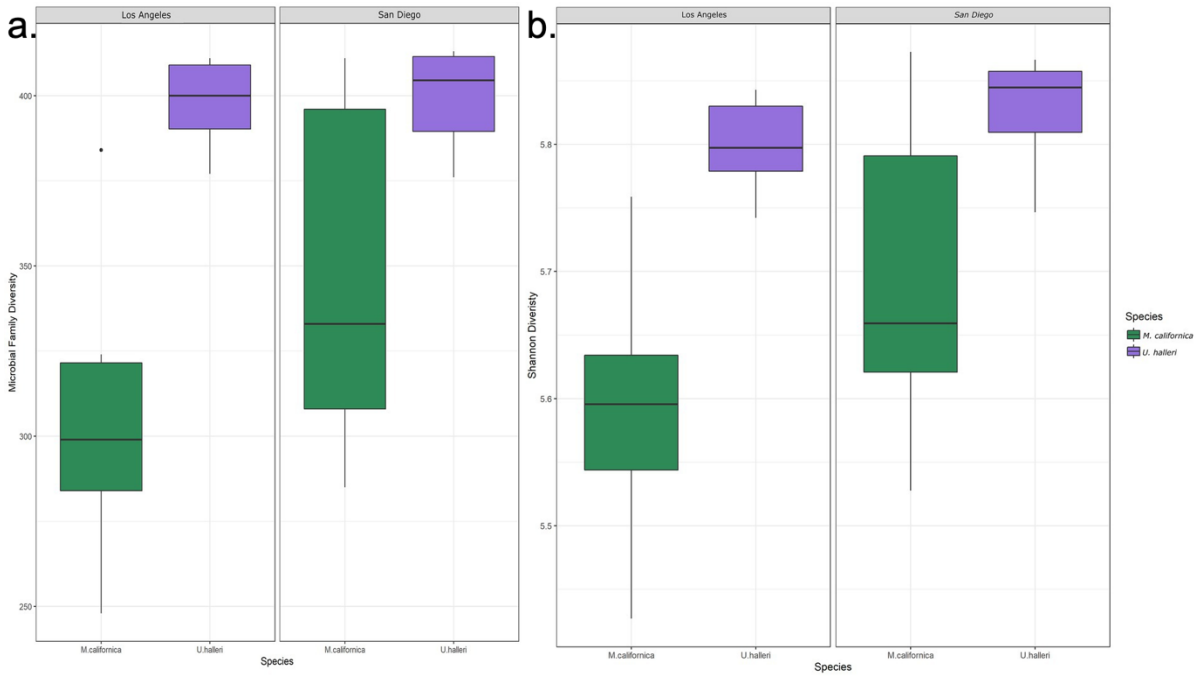

Figure 3.2. Boxplots depicting the differences in a.) total number of microbial families and b.) Shannon diversity between host species and location.

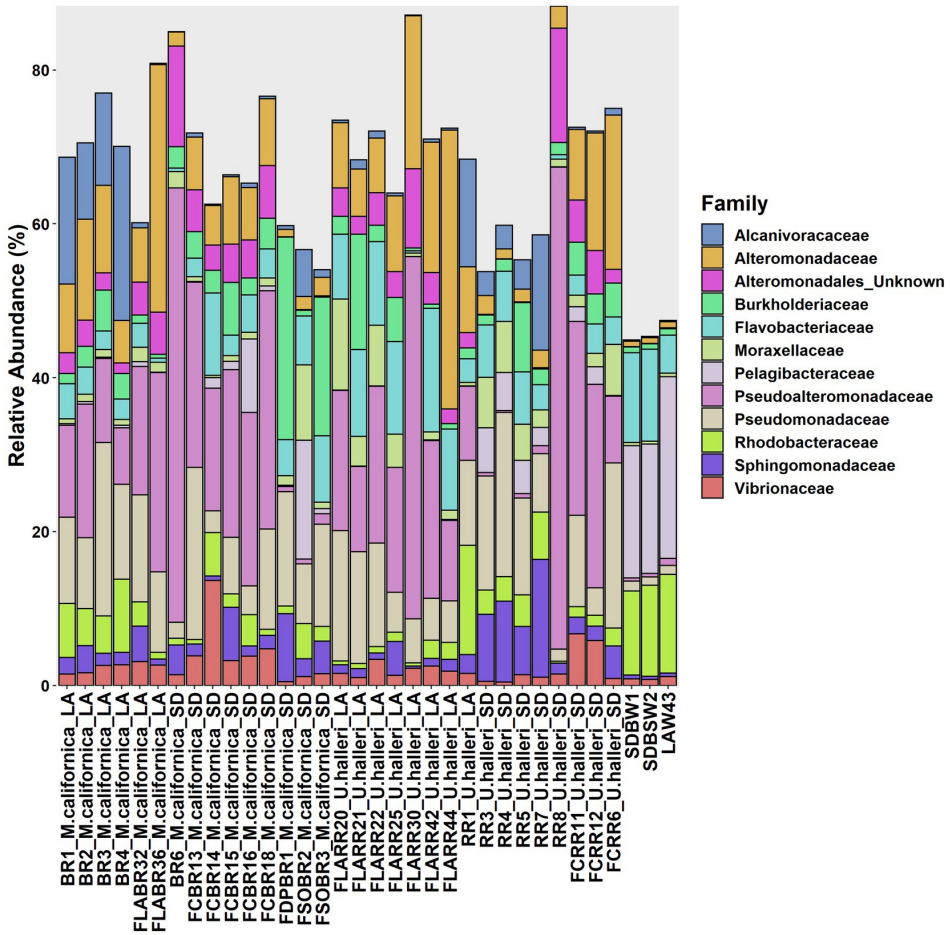

Figure 3.3. Variation of microbial families across *M. californica*, *U. halleri* and seawater microbiomes. Rare taxa are excluded from this graph, only microbes present with a relative abundance of 10% or greater in at least one sample are included. Samples appear in the same order as Supplementary Table 3.1.

Myliobatis californica and *U. halleri* microbial families were significantly different from each other and the water column, indicating species specificity (PERMANOVA $p = 0.001$, $df = 2$, Pseudo-$F = 5.449$). *M. californica* microbiomes were not significantly different between San Diego and Los Angeles (PERMANOVA $p = 0.123$, $df = 1$, Pseudo-$F = 1.4083$). In contrast, *U. halleri* microbiomes were significantly different between the two locations (PERMANOVA $p = 0.019$, $df = 1$, Pseudo-$F = 3.8433$) (Figure 3.4). *M. californica* and *U. halleri* microbiomes were 15.84% dissimilar to each other and

Pseudoalteromonadaceae was the highest contributor to differences between hosts contributing 1.29% of the difference between microbiomes. *M. californica* microbiomes had greater variance than *U. halleri* microbiomes (PERMDISP p = 0.003, F = 19.58, df = 2) (Table 3.1; Figure 3.4), which was confirmed with a SIMPER analysis showed *M. californica* had an 82.67% taxonomic similarity between individuals, whereas *U. halleri* were 88.56% similar between individuals.

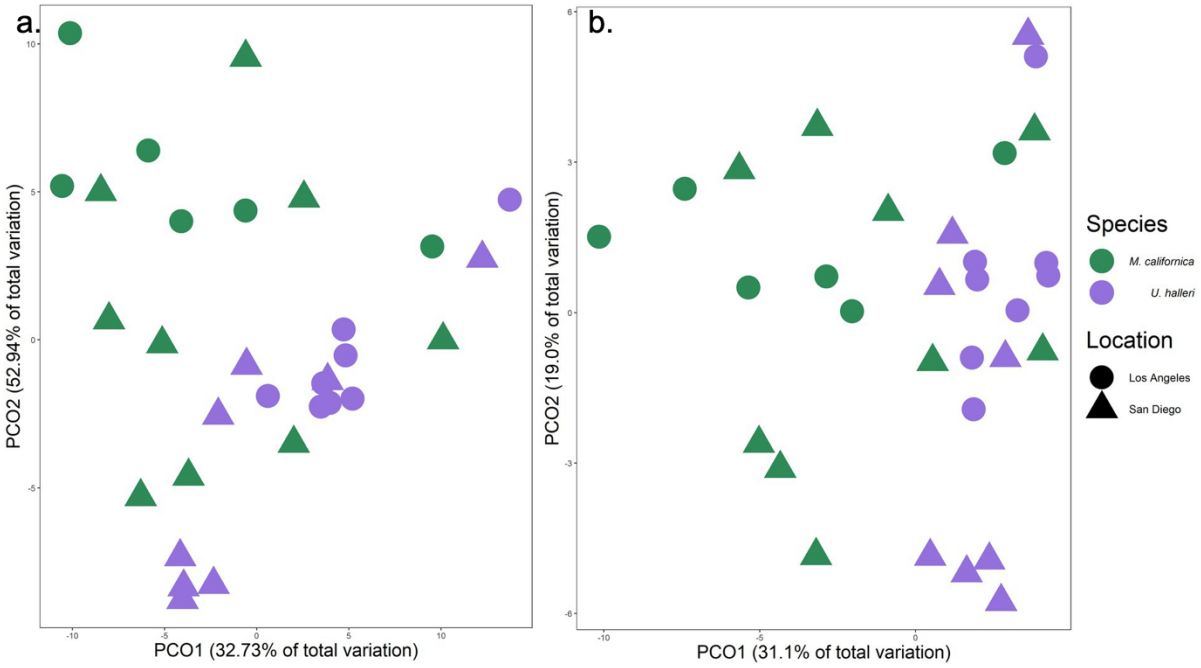

Figure 3.4. Principal coordinate analysis of Bray–Curtis similarity between a.) taxonomic composition and b.) functional gene potential (SEED Level 3 Subsystems) of *M. californica* and *U. halleri* microbiomes across sampling locations, showing the variation between the two species microbiome and the larger variation in microbiome that occurred across the individual *M. californica*.

Table 3.1. Pairwise PERMDISP comparisons between host species from each location.

Microbial Family Pairwise Comparisons	t	*P perm*	SEED Level 3 Function Pairwise Comparisons	t	P perm
M. californica Los Angeles, *M. californica* San Diego	0.36	0.751	*M. californica* Los Angeles, *M. californica* San Diego	0.27	0.826
M. californica Los Angeles,	1.88	0.166	*M. californica* Los Angeles,	1.75	0.185

U. halleri San Diego			*U. halleri* San Diego		
M. californica Los Angeles, *U. halleri* Los Angeles	3.63	0.012	*M. californica* Los Angeles, *U. halleri* Los Angeles	3.84	0.003
M. californica San Diego, *U. halleri* San Diego	2.96	0.035	*M. californica* San Diego, *U. halleri* San Diego	2.82	0.024
M. californica San Diego, *U. halleri* Los Angeles	5.61	0.002	*M. californica* San Diego, *U. halleri* Los Angeles	6.10	0.001
U. halleri San Diego, *U. halleri* Los Angeles	1.56	0.355	*U. halleri* Los Angeles, *U. halleri* San Diego	2.29	0.088

Functional potential

The functional potential (SEED Level 3 Subsystems) of the skin microbiome was different between host species (PERMANOVA $p = 0.001$, $df = 1$, Pseudo-$F = 5.0761$). Neither ray species had significantly different SEED Level 3 functional potential between locations (PERMANOVA $p = 0.121$, $df = 1$, Pseudo-$F = 1.4239$ and $p = 0.059$ $df = 1$, Pseudo-$F = 2.254$ for *M. californica* and *U. halleri* respectively). SIMPER showed the functional potential of *M. californica* microbiomes were 89.56%, and *U. halleri* microbiomes were 93.16% similar. *M. californica* microbiome functions were 16.18% and *U. halleri* microbiomes were 15.15% dissimilar to the water column microbes. Host microbiome functional potential were 10.03% dissimilar. *Myliobatis californica* has significantly higher variance within the microbiome than *U. halleri* (PERMDISP $p = 0.002$, $df = 3$, $F = 9.74$). SIMPER analysis identified RNA Polymerase III-like genes accounting for the greatest difference (0.69%) between host microbiome functional potential., Out of 1,243 Level 3 functional genes, 18 have a relative abundance of ≥1% in at least one sample and vary across rays and water column. Bacterial chemotaxis, bacterial hemoglobin, cobalt-zinc-cadmium resistance, copper homeostasis, flagellum, multidrug resistance efflux pumps, RNA polymerase III-like, and Ton and Tol transport system genes are overrepresented in host microbiomes compared with the water column microbes (Figure 3.5). All high abundance

gene pathways were significantly different between *M. californica* and the water column (ANOVA $p < 0.05$, Tukey–Kramer $p < 0.05$) except respiratory complex I (Tukey–Kramer $p = 0.3$) and terminal cytochrome C oxidase (Tukey–Kramer $p = 0.06$). *Urobatis halleri* microbiomes had significantly different pathways from the water column (ANOVA $p < 0.05$, Tukey–Kramer $p < 0.05$) except RNA Polymerase III-like and terminal cytochrome C (Tukey–Kramer $p = 0.98$ and $p = 0.96$ respectively) (Figure 3.5; Supplementary Table 3.2).

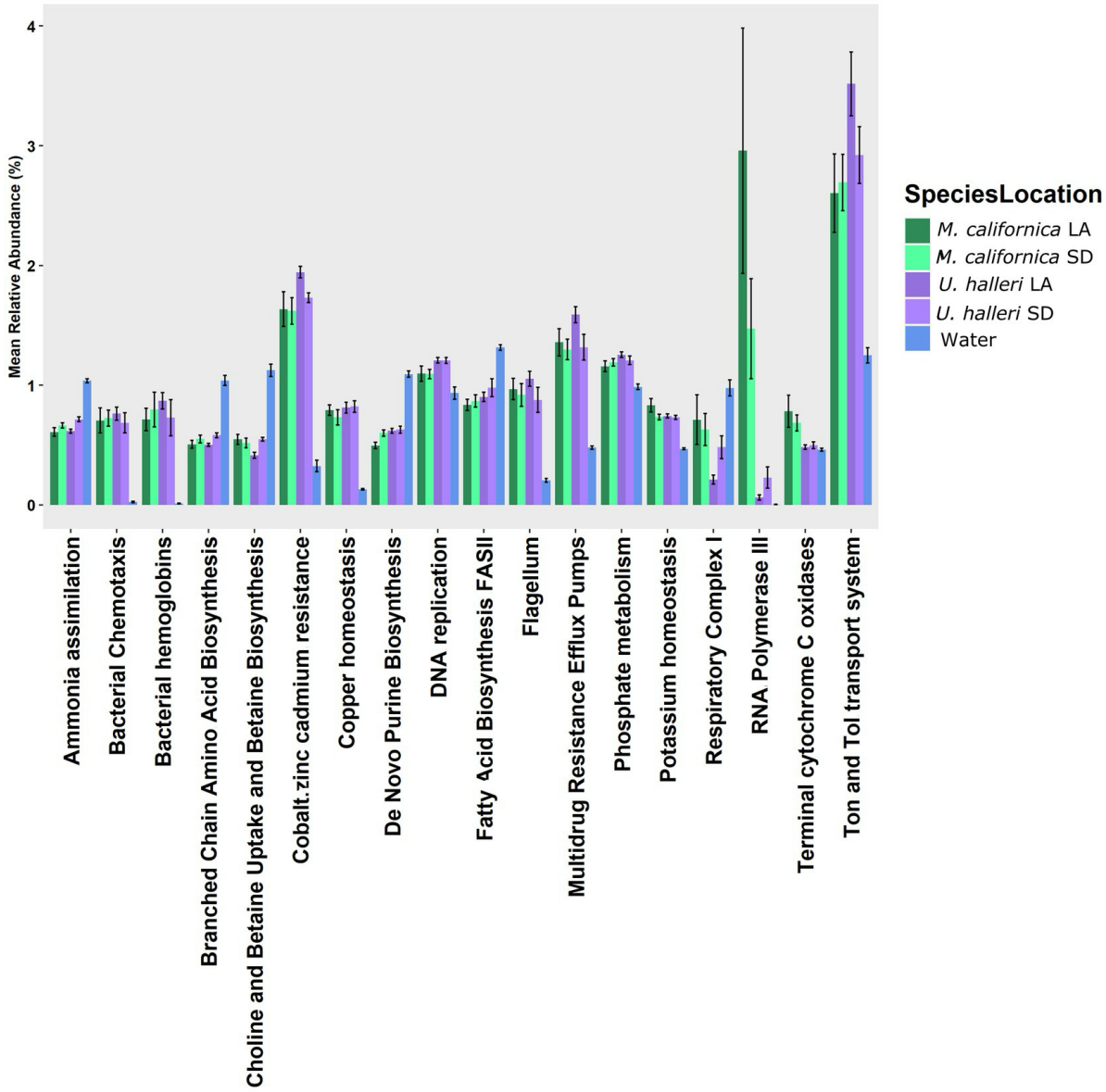

Figure 3.5. The relative abundance of functional genes (Level 3 SEED Subsystems) with >1% that showed a variation with the water column microbes.

Metagenome Assembled Genomes

Across all stingray and water microbiomes, cross assembly of 34 metagenomes yielded five high-quality MAGs (Bins 9, 16, 17, 31, and 33) spanning a range of bacterial phyla. Bin 9 featured an 84.5% complete genome 2,998,016 bp in length from 679 contigs, with 3.14% contamination and 33.33% strain heterogeneity. Bin 16 featured a 74.14%

complete genome 2,866,836 bp in length from 470 contigs with 0 contamination and 0 strain heterogeneity. Bin 17 featured an 86.13% complete genome 3,699,146 bp in length from 136 contigs with 0.84% contamination and 33.33% strain heterogeneity. Bin 31 featured a 93.4% complete genome 2,583,396 bp in length from 514 contigs with 3.27% contamination and 72.5% strain heterogeneity. Bin 33 featured an 89.94% complete genome 2,371,543 bp in length from 299 contigs with 4.69% contamination and 93.33% strain heterogeneity (Supplementary Table 3). Three bins (16, 17, 33) were > 95% similar to existing genomes, the remaining two (Bin 9 and 31) are novel species <95% similar to existing genomes (Supplementary Table 3.3).

Functional pathways (SEED Subsystem: Level 1) featured a high proportion of active metabolism genes (39.03% ± 5.17) across all five MAGs. Utilization of monosaccharides (0.88% ± 1.74), di- and oligosaccharides (0.02% ± 0.03), and sugar alcohols (0.08% ± 0.17) occurred in low abundance across species and were most abundant in Rhodobacteraceae (Figure 3.6). In contrast, utilization of more complex polysaccharides was present in all species excluding *Rhodobacteraceae* (0.26% ± 0.34) (Figure 3.6). *Rhodobacteraceae* and *Moraxellaceae* also showed higher levels of ribosome biogenesis (Figure 3.6). DNA repair (3.83% ± 1.07), central metabolism (6.88% ± 1.74), and RNA processing and modification (5.44% ± 1.54) were among the most abundant active genes across species, with greater abundance in *Alcanivoracaceae*, Gammaproteobacteria, and *Rhodobacteraceae* bins (Figure 3.6). Furthermore, pathways involved in stress response, defense, and virulence were abundant (8.58% ± 0.36), including specific genes related to heat/cold shock (1.59% ± 0.13), osmotic stress (1.09% ± 0.47), resistance to antibiotic and

toxic compounds (2.88% ± 0.78) and multidrug efflux systems (0.53% ± 0.30) were also active.

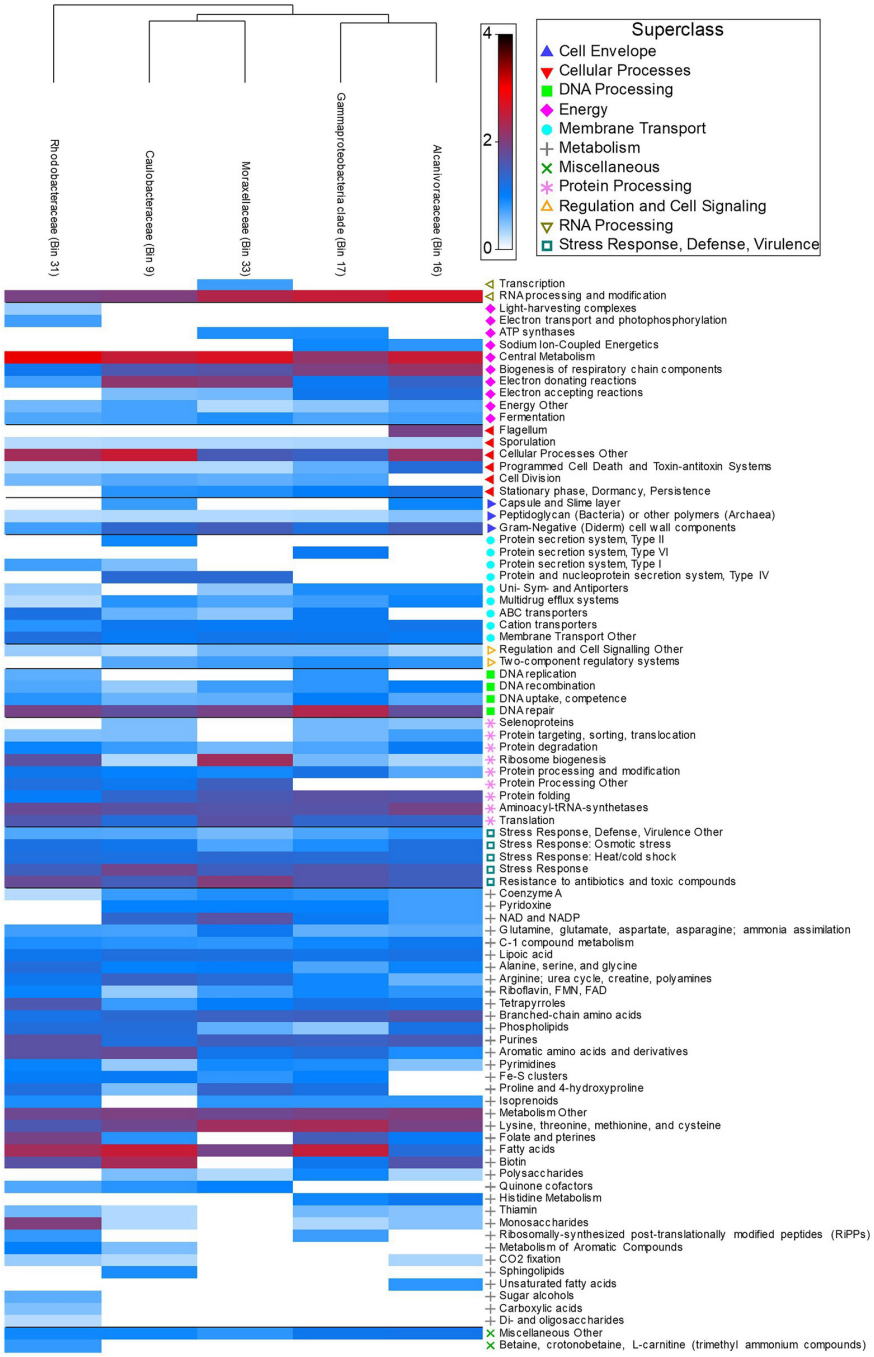

Figure 3.6. Heatmap depicting relative abundance of functional genes (Level 3 Subsystems) grouped into broader Level 1 Superclass present in host associated MAGs.

Discussion

We demonstrated that *Myliobatis californica* (bat ray) and *U. halleri* (round ray) microbiomes are species-specific, and distinct from the water column, regardless of sampling location showing that host phylogeny is an important selection pressure for the microbiome. Consistent with epidermal microbiomes of Elasmobranchs including *Alopias vulpinus* (thresher sharks), *Triakis semifasciata* (leopard sharks), *Rhincodon typus* (whale sharks), and *Aetobatus narinari* (spotted eagle rays), which show a pattern of species specificity and host selection (Doane et al., 2017, 2022; Larsen et al., 2013; Storo et al., 2021). The microbes selected by *M. californica* and *U. halleri* were from the Proteobacteria phylum including *Pseudoalteromonadaceae*, *Alcanivoracaceae*, and *Pseudomonadaceae* which is consistent with other ray species such as *Rhinoptera bonasus* (cownose ray), *Gymnura altavela* (butterfly ray) and *Dasyatis hypostigma* (groovebelly ray) (Gonçalves e Silva et al., 2020; Kearns et al., 2017). Cultured isolates from *R. bonasus* mucus include *Pseudoalteromonas sp*., *Alteromonas sp*., and *Vibrio sp*. which are recovered in our metagenomes (Ritchie et al., 2017). *Myliobatis californica* microbiomes have a significantly higher intra-species microbiome variance than *U. halleri*, suggesting fine-scale feature of the host epidermis and potential mucus turnover is affecting microbiome structure.

Myliobatis californica microbiomes are variable and show no significant difference in microbial taxonomy or functional potential across locations. *M. californica* are dispersed along the California coast and migrate up to 259 km during the summer to mate (Gong, 2022). Traveling large distances may obscure location specific effects on the microbiome.

However, the microbial richness of the *M. californica* was lower compared with *U. halleri* suggesting selection via skin characteristics rather than migrative behavior. We suggest that the highly variable microbiome is associated with mucus production. We observed large amounts of mucus on *M. californica*, and while features of this mucus have not been measured specifically, mucus is consistently being produced and sloughed off as marine organisms propel through the water (Parrish and Kroen, 1988). *Manta birostris* (giant manta ray) and *T. semifasciata* (leopard shark) mucus has high isotopic turnover compared to other tissues, suggesting a highly variable environment for microbes (Burgess et al., 2018; Malpica-Cruz et al., 2012). Soluble fractions of sea bream (*Sparus aurata*) mucus had high carbon isotope turnover within 12 h of a diet switch, suggesting that soluble fractions of mucus are continuously produced and shed (Ordóñez-Grande et al., 2020). High turnover of mucus serves as a selective pressure, and only microbes that are adapted to replicate quickly would be able to survive causing the microbiome to have lower diversity and higher intraspecies variation (Figures 3.2, 3.4).

The microbiome characteristics of *U. halleri* suggests the mucus and epidermis condition are different to *M. californica*. The *U. halleri* microbiomes were consistent across individuals and were location specific. *U. halleri* migrate shorter distances (about 30 km) and have small home ranges but maintain high gene flow across southern California (Plank et al., 2010). Thus, low gene flow and genetic drift between the host from different locations that then modifies the microbiome is not likely to be the cause of the differences in microbiomes (Nemergut et al., 2013). Similar to *U. halleri*, the microbial taxonomic composition in *Carcharhinus melanopterus* (black tip reef sharks) was location specific across five reef sites (Pogoreutz et al., 2019). *C. melanopterus* also have small home ranges

compared to pelagic shark species, and transfer microbes between individuals during feeding and mating (Mull et al., 2010; Pogoreutz et al., 2019). Location specific diet affected the gut microbiomes of detritivores feeding fish (Wu et al., 2012) and could be a feature of the *U. halleri* microbiomes. Divergent selection pressures between locations, such as interactions with the water column microbes, which show biogeography (Haggerty & Dinsdale, 2017) or physicochemical variables could play a role. The skin-microbiomes of three fish species in the Amazon identified high degree of co-correlations between skin and water column microbes, but very few co-correlations between the skin microbes and physiochemical variables suggesting that the host is filtering a sub-selection of the microbes in the surrounding (Sylvain et al., 2020). Despite significantly different microbial taxonomic abundances, microbial family diversity and Shannon diversity were maintained at both locations. Therefore, we suggest that turnover rate of the mucus of *U. halleri* is lower than *M. californica* and the skin microbiomes may be interacting with the water-column microbes, similar to teleost fish.

The epidermis and mucus production of sharks, rays, and teleost fish is variable and affects the microbiome. Sharks have minimal mucus and dense coverage of denticles (Meyer & Seegers, 2012), which leads to a highly structured microbiome (Doane et al., 2017, 2022). Teleost fish have epidermal scales which are covered with mucus which is reflect in microbiomes that has high alpha diversity high variability between individuals of the same species (Chiarello et al., 2015, 2018). Proteases in stingray mucus have antibacterial and antifungal activities, but the effects of stingray antimicrobial proteins on microbiome composition have not been explored (Vennila et al., 2011). In fish, the skin mucus microbes are species- specific in nature, but interaction networks showed high connectivity between

the fish and water column microbes, suggesting they are affected by the microbes and environmental features of the location (Sylvain et al., 2020). Elasmobranchs demonstrate phylosymbiosis in their epidermal microbiomes, but signals are weak or absent in mucus microbiomes of fish, suggesting that mucus is more influenced by environment than denticle covered surfaces (Chiarello et al., 2018; Doane et al., 2020; Pollock et al., 2018).

Neither *M. californica* nor *U. halleri* had significantly different functional potential between locations, suggesting functionally redundancy (Louca et al., 2018). Functional redundancy describes that metabolic functions can be carried out by taxonomically distinct microbes (Louca et al., 2018). *Triakis* semifasciata (leopard shark) microbiomes maintain functional redundancy throughout time, even with taxonomic fluctuations (Doane et al., 2022). Functional genes present in high abundance on the stingrays, such as heavy metal resistant genes, Ton and Tol transporters, relative to the water column are consistent with other shark metagenomes (Doane et al., 2017, 2020). *Triakis* semifasciata and *A. vulpinus* (thresher sharks) both had higher relative abundance of cobalt zinc and cadmium resistance, and Ton and Tol transport system genes compared with the surrounding water column (Doane et al., 2017, 2022).

An RNA Polymerase III- like gene was highly abundant in *M. californica* microbiomes compared to both *U. halleri* and the water column. Our bioinformatic pipeline compares the stingray metagenomes to Chondrichthyan host genomes (< 10% of reads removed before microbial annotation), thus removing the possibility that host contamination was contributing to the presence of the high relative abundance of RNA Polymerase III-like genes in the metagenomes. The RNA Polymerase III-like gene is a eukaryotic specific gene but shows similarity with other RNA Polymerase subunits (i.e., I and II) in prokaryotes and

viruses (Allison et al., 1965; Sweetser et al., 1987) and thus we suggest the RNA-Polymerase III-like gene is of prokaryote origin, but divergent Polymerase genes that are currently represented in the database. This is consistent with the high novelty that was identified in the MAGs that we constructed from the stingray metagenomes. In Saccharomyces, RNA Polymerase III is active in the presence of abundant nutrients, leading to rapid growth, whereas in nutrient depleted environments RNA Polymerase III activity declines (Roberts et al., 2003). Growth rate due to genetic variation is not well understood but has been correlated with high copy numbers of ribosomal RNA operons (*rrn*) (Ciara et al., 1995; Klappenbach et al., 2000). High *rrn* copy numbers in a bacterial isolate from high nutrient marine environment suggests a link between RNA genes and adaptations to high nutrient conditions (Lauro et al., 2009). Therefore, we suggest in a nutrient rich mucus layer of the stingrays, microbes are growing rapidly, which is reflected in a high relative abundance of RNA Polymerase genes. These genes constituted <1% of the genes in microbiomes from *T. semifasciata*, *R. typus*, *A. vulpinus*, and *C. carcharias* (great white shark) (Doane et al., 2017; Goodman et al., 2022; Pratte et al., 2022). Shotgun microbiome studies of teleost fish are currently limited to the gut (Legrand et al., 2020), thus making comparison with fish epidermal microbiome not possible. High relative abundance of RNA processing genes only in Elasmobranchs with mucus and the high proportional abundance in the *M. californica* microbiome suggests mucus production and turnover are important structural feature of skin microbiomes and warrant future investigation.

Stingray MAGs had high completeness and low contamination but could only be annotated to the family level, highlighting novel bacterial species. The construction of MAGs identified *Moraxellaceae* and *Rhodobacteraceae*, both of which have been observed

in captive *R. bonasus* (cow-nose rays) (Kearns et al., 2017). Functional gene pathways including RNA processing, metabolism, and antimicrobial pathways were abundant in stingray MAGs. The RNA processing genes present in the MAGs were described as "active" by the PATRIC database algorithms and were highly similar to RNA genes in the NCBI database, supporting the single read data. The thick mucus layer on the batoids epidermis provide a high nutrient matrix for microbial growth (Shoemaker and LaFrentz, 2015) and while mucus properties were not measured, our data suggests variation in mucus turnover rate between the two stingray hosts. Antimicrobial genes present in MAGs signify interspecies competition within the stingray microbiomes consistent with competitive interaction of the microbes cultured from stingray mucus (Gonçalves e Silva et al., 2020; Kearns et al., 2017; Ritchie et al., 2017). The batoid mucus shows antibacterial action against human pathogens and expedites the healing processes of host wounds (Gonçalves e Silva et al., 2020; Ritchie et al., 2017). The ubiquitous presence of antimicrobial genes across the MAGs raises the question of whether the antibiotic properties of the stingray mucus is being produced by the host or the microbial community. Multidrug resistance efflux pumps within the microbial genome provides resistance to antimicrobials (Piddock, 2006; Vila and Martinez, 2008; Li X.-Z. et al., 2015; Jang, 2016) and these are common in other Elasmobranch microbiomes (Doane et al., 2017, 2022). A high abundance of antimicrobial resistance genes reflects the elevated abundance of antibiotics and toxic compounds within the mucus and the interspecific competition within the microbial community.

The microbiome of *M. californica* maintains taxonomic and functional stability across southern California. *Urobatis halleri* maintain functional gene potential but have

significantly different taxonomy across locations despite high similarity between individuals. While the microbiome of the rays shared many characteristics with other Elasmobranch species, the variation in β-diversity across ray species suggest variation in mucus turnover rates may be an important structuring feature of epidermal microbiomes and requires further investigation. Host microbiomes enriched in heavy metal resistance genes appears to be a signature of Elasmobranch microbiome and may suggest changes in host health. The high levels of RNA Polymerase pathways, a signature of rapid microbial replication, combined with the high levels of antimicrobial resistance suggests stingray mucus promotes microbial competition.

CHAPTER 4: INTERACTIONS OF MUCUS MONOSACCHARIDES AND THE EPIDERMAL MICROBIOME IN FOUR BENTHIC ELASMOBRANCHS

Context

Fish mucus provides the first defence against environmental pathogens and harbors the commensal microbiome. Mucus is largely made up of chains of polysaccharides attached to proteins called glycoproteins or glycans. Glycoprotein composition varies across organisms and plays a crucial role in modulating microbial communities. Glycan structures and composition in Elasmobranchs have only been studied in three species (Bachar-Wikstrom et al., 2023a, 2023b) and have never been linked to microbial metabolism. Mucus glycans have only been analysed in captive hosts, and samples from multiple individuals were "pooled" to achieve enough biomass for analysis. Here I expand what is known about shark mucus composition by analysing the skin mucus carbohydrate composition of four species of wild Elasmobranch. I also make the first connection between Elasmobranch mucus and microbial community functions. This work is under review in Environmental Microbiology Reports.

Abstract

Epidermal mucus is a complicated mixture macromolecules which acts as the first line of defence for organisms against abrasions and infections. We quantified the carbohydrate (monosaccharide) composition of the mucus from four Elasmobranchii hosts, including eagle rays (*Myliobatis tenuicaudatus*), Port Jackson sharks (*Heterodontus portusjacksoni*), Australian angelsharks (*Squatina australis*), and whitespotted skates (*Dentiraja cerva*). Elasmobranchii had low amounts of mucus and low proportion of

carbohydrates (<10 %) compared with other marine organisms. Four key monosaccharides; glucose, glucosamine, galactose, and fucose, were identified in mucus samples. Hosts exhibited distinct, species-specific monosaccharide signatures. We identified key carbohydrate microbial genes from host and water microbiomes. Elasmobranch microbiomes had a higher relative abundance of carbon utilization genes compared to the water column and contained gene pathways for the utilization specific monosaccharides found in host mucus, suggesting that the host mucus was a regulator of the microbiome. Elasmobranch epidermal microbiomes had the genetic machinery required for detecting, transporting, and metabolizing monosaccharides and other carbohydrates present in the host mucus, demonstrating the selective nature of Elasmobranch epidermal mucus.

Introduction

Skin and mucus provide the first defence for all organisms against abrasions, infections, and buffers environmental changes (Meyer & Seegers 2012). A key difference between marine organisms, such as fish and sharks, and many terrestrial organisms, is that their skin lacks a dead, keratinized protective layer. Since fish and shark epidermis consists entirely of living cells the protection from the mucus is paramount (Meyer & Seegers 2012). In marine environments the mucus is constantly in contact with the surrounding water and is continuously bombarded by chemicals, microbes, and debris.

Mucus is coded for by mucin genes (Muc gel-forming mucus proteins) that occur across vertebrate and invertebrate hosts and these genes code for the backbone of mucus molecules (Lang et al., 2016). While these genes are consistently present in all vertebrate organisms, the homologs vary in Chondrichthyans. The chimera, *Callorhinchus milii* (Australian ghost shark), has Muc19, Muc2, and Muc5 which are consistent across

vertebrates, however two orthologs of Muc6 gene are present, even though these are lost in Osteichthyes or ray-finned fishes (Lang et al., 2016). Mucus is secreted by goblet cells located on the epithelial surface (Dash et al., 2018). Sacciform and club cells secrete additional molecules that interact with the mucin to form a range of glycoproteins (Reverter et al., 2018). Glycoproteins are proteins covalently bonded to oligosaccharides (chains of multiple monosaccharides) usually by either an asparagine amino acid (N-glycosylation); or serine or threonine amino acids (O-glycosylation) (Dell and Morris 2007). O-glycans are more common in vertebrates and are classified into Core types 1-8 which are made of different configurations of monosaccharides. Core 1 is composed of galactose attached to N-Acetylgalactosamine (GalNAc). Adding N-Acetylglucosamine (GlcNAc) to a core 1 glycan creates a core 2 glycan. Cores 3-8 are composed of the same monosaccharides arranged in different configurations (Watson et al., 2015). The mucin O-glycan profile of ray-finned fish species is well characterized (Benktander et al., 2021, Jin et al., 2015, Thomsson et al., 2022, 2024), including rainbow trout, which contained 54 glycan types with nine monosaccharide residues. Core 1, 2, 3, and 5 were common, with shorter chains present on the skin compared with other organs (Thomsson et al., 2022).

Mucus provides mechanical protection from the environment by acting as a physical barrier. For example, sponge mucus traps debris that is purged via peristaltic “sneezing” contractions (Kornder et al., 2022) and coral mucus efficiently traps and transports particles during feeding (Brown & Bythell 2005). Seastar (*Marthasterias glacialis*) glycoproteins (proteins with covalently bonded carbohydrate groups) inhibit bacteria from adhering to glass slides by creating clumps of bacteria that are dislodged by flowing water (Bavington et al., 2004). Fish mucus is continuously sloughed during swimming and the rate of mucus loss

is variable. Detectible concentrations of mucus are shed from schooling fish (*Menidia menidia*) at fast swimming speeds (Parrish and Kroen 1988). Stable isotope analysis of mucus and muscle showed a higher turnover of mucus than muscle tissue in giant manta ray (*Manta birostris*) (Burgess et al., 2018) suggesting mucus is lost during swimming. Internally, the intestinal mucus of fish functions as a barrier that bacterial-sized particles cannot penetrate (Sharba et al., 2022). The sloughing of mucus in response to physical interactions and provides hosts with protection from their environment.

Mucus contains relevant immune molecules which provide chemical protection from bacterial invasion. Proteases cleave microbial proteins which damages microbes and inhibits infection of the host (Rawlings 2013). Lysozymes target the bacterial cell wall and works in tandem with other immune molecules to modulate response to infections (Ragland and Criss 2017). Lectins are proteins that bind to foreign carbohydrates on viruses or bacteria to cause immobilization before destruction by macrophages (Dash et al., 2018; Santos et al., 2014). Antimicrobial peptides are a broad category of innate immune molecules that target pathogens directly or regulate host immune response (Zhang & Gallo 2016). Mucus and symbiotic bacteria isolated from stingrays have antibiotic activity against *Bacillus subtilis* and other potentially pathogenic bacteria (Luer 2014). Glycoproteins, despite not being classified as immune molecules, also provide chemical protection to the host. Oligosaccharides are added to secreted proteins post-translationally, increasing the diversity of immune-relevant glycoproteins (Ouwerkerk et al., 2019). Fucose is prevalent in glycoproteins and plays important roles in host immune systems (Thomès & Bojar 2021). Fucose-containing glycans were abundant in shark and skate epidermal mucus, but their role in shark immune response is unknown (Bachar-Wikstrom et al., 2023a). Elasmobranch skin

mucins have larger glycans with fucose residues compared to salmon skin, suggesting that shark glycans may have different roles, one of which may be providing nutrients to beneficial bacteria (Bachar-Wikstrom et al., 2023a; Padra et al., 2013).

Glycan structures are variable across phylogenetic lineage, as glycans evolve rapidly to evade pathogens and general microbial degradation (Bishop & Gagneux 2007). However, while protecting organisms from invasion of pathogens, glycans also attract and harbor beneficial microbes and may structure the microbiome. In the human gut, beneficial microbes outcompete pathogens for host glycans and prevent pathogens from infecting the host (Pickard & Chervonsky 2015). The different glycosylation patterns on skin compared with the intestine of fish prevented the pathogens binding on the skin, while encouraging binding and immobilization of pathogens in the intestine (Thomsson et al., 2022). Bacteria co-varied with glycans in gilthead seabream mucus (Thomsson et al., 2024), but the mechanism for host glycan-microbiome relationship is unexplored in Elasmobranch mucus.

Mucin profiles of Elasmobranchs have been investigated on three species only (Bachar-Wikstrom et al., 2023a). The spiny dogfish (*Squalus acanthias*) and little skate (*Leucoraja erinacea*) had 39 and 22 glycans, respectively. Dogfish and little skate mucus was made primarily of O-glycans, with a small presence of N-glycans. Spiny dogfish exhibited a diverse set of cores 1 and 2 O-glycans, with a high prevalence of fucose. These glycans were mostly neutral and lacked significant sialylation or sulfation. Little skates, however, had a higher proportion of acidic O-glycans, with substantial sulfation and sialylation with N-acetylneuraminic acid. The third species, the chain catsharks (*Scyliorhinus retifer*) yielded very few glycans, but those detected included core 2 glycan structures and a rare poly- N-acetylhexosamine glycan in dogfish, which are unusual outside

of bacteria and invertebrates (Bachar-Wikstrom et al., 2023a). Therefore, the unique glycan composition of Elasmobranch mucus, differs significantly from ray-finned fish and may have functional implications in host-microbe interactions and immune defense.

Skin microbiomes of three Elasmobranchii clades, including Division Selachii (sharks), and Division Batomorphi (rays and skates), across 20 species are species-specific and follow host phylogeny (Doane et al., 2020; Perry et al., 2021). The microbiomes of sharks have low intraspecific variation, whereas the microbiomes of rays are more variable across individuals of the same species which may reflect high turnover of mucus (Doane et al., 2017; Kerr et al., 2023). Gram-negative bacteria including Pseudomonas, Vibrio, Pseudoalteromonas, and Psychrobacter are abundant on shark and ray skin despite these taxa being less abundant in the water column indicating the selective nature of both denticle and mucus skin types (Black et al., 2021; Doane et al., 2022; Pogoreutz et al., 2019). Elasmobranch evolutionary history, unique physiology, and ecological importance has identified this clade for microbiome exploration to identify eco-evolutionary patterns (Doane et al., 2020; Pratte et al., 2022), niche microenvironments (Black et al., 2021; Clavere-Graciette et al., 2022; Pogoreutz et al 2024,), and conservation efforts (Goodman et al., 2023; Perry et al., 2021). Linking epidermal physiology with the microbiome will provide insight on how host and microbe interact and inform predictions for how this relationship might change in response to climate, pollution, diet and other factors. Because of the lack of information on shark mucus chemistry and physical properties, the link between host physiology (mucus chemistry) and microbial community composition is difficult to resolve. Pattens in mucus chemistry (including monosaccharides) can be used as indicators for health, evolution, and response to environmental change.

Our study makes the first connections between mucus chemistry (monosaccharide composition) and microbial gene functions. Here we describe the mucus monosaccharide composition of four species of Elasmobranchs, eagle rays (*Myliobatis tenuicaudatus*), Port Jackson sharks (*Heterodontus portusjacksoni*), Australian angelsharks (*Squatina australis*), and whitespotted skates (*Dentiraja cerva*). We identify carbohydrate-specific microbial functions using metagenomics to identify the interactions between the microbiomes and Elasmobranch mucus. We demonstrate that Elasmobranch epidermal microbes have the metabolic potential to digest host-specific monosaccharides which highlights the bacterial adaptation to the Elasmobranch host environment.

Methods

Sample Collection

We attempted epidermal mucus collection from seven Elasmobranchs in South Australia. Eagle rays were collected using a seine net at Seacliff Beach, South Australia. Angelsharks, whitespotted skates, Port Jackson sharks and one eagle ray were caught opportunistically during observer prawn trawls conducted by the South Australian Research and Development Institute (SARDI) (McLeay & Hooper 2024). The observer trawls are conducted twice a year and during this time only a single net is deployed without an exclusion device, where some Elasmobranchs are caught as bycatch (Burnell et al., 2015). The trawls are deployed for 30 minutes at varying depths and locations in the Gulf St. Vincent, South Australia. Upon trawl retrieval, Elasmobranchs were sorted from the rest of the catch and placed into separate tubs filled with seawater for sampling before returning them to the ocean (AEC BIOL4067-10).

Metagenomes were collected by filling a “supersucker” or blundt-end-two-way syringe with sterile seawater as described in Kerr et al., 2023. The microbial slurry was filtered through a 0.22 μm Sterivex™ filter (Merck, Australia) and stored on board at -45°C before being transported to the lab for DNA extraction. Seawater microbiomes were collected from various locations in the Gulf St. Vincent (Supplementary Table 4.2). One liter of seawater was filtered through a 0.22 μm Sterivex and kept on ice until being stored long term at -20°C.

After microbial collection, mucus was scraped from the dorsal surface of the shark or ray. Multiple techniques of collection were trialed because Elasmobranchs are covered in dermal denticles and have low mucus volume. Scraping devices such as glass slides or credit cards – which are commonly used during the collection of mucus from fish (Fernandez Cunha et al., 2024), but the raised dermal denticles stopped the scraping device from contacting the skin surface and therefore, prevented mucus collection. Therefore, we developed a method that used textured polyurethane coated gloves (Safety Culture Marketplace) that adhered to mucus and the gloved hand could follow the contours of the shark/ray’s body to scrap off the mucus. The plastic-gloved hand collected between 2 – 4 ml of mucus from most organisms (Supplementary Table 4.1). Mucus was frozen on board at -45°C and transported to the laboratory on ice before being frozen in liquid nitrogen and stored at -80°C.

Carbohydrate Analysis

Mucus samples were prepared for oligosaccharide analysis by freeze drying and desalting. First the mucus was weighed (wet weight) and prepared for analysis by freeze drying (-80°C) for 72 hours and reweighed when dried to provide an estimate of the amount

of solid material within the mucus. The high salt content of the mucus required the samples to undergo de-salting to ensure the integrity of the instruments. Mucus samples were added to Microcon Centrifugal Filters (10kDa) (Merck, Australia) with 500 uL of Ultra-pure nuclease-free water (0.1-µm filtered) (Invitrogen) and spun at 14,000 RCF for 12 minutes, flow through was discarded and a second rinse was repeated. The columns were inverted into a fresh 1.5 mL tube and spun at 14,000 rcf for 3 minutes to retain the mucus concentrate. Mucus concentrate was freeze-dried again, the total biomass dry weight was recorded.

Five milligrams of freeze-dried samples was used for monosaccharide analysis as described in Comino et al., (2013). Where sample mass was low, samples from multiple individuals of the same species was combined to achieve five milligrams. Briefly, samples were hydrolyzed (cleaving the polysaccharides into monosaccharides) with 1 mL of 2M trifluoroacetic acid and incubated at 100°C for three hours. Samples were cooled and diluted by adding 40 µL of each sample to 360 µL of water (1:10 dilution). Diluted hydrolysate was derivatized using 1-phenyl-3-methyl-5-pyrazolone (PMP) at 70 ºC for 1 h. Monosaccharide profiling was conducted using the Agilent 1260 Infinity II High-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD). Ten µL of derivatized samples was inject to a Phenomenex Kinetex C18 analytical column (3 × 100 mm 2.6µm 100Å). The monosaccharides were eluted at 30 ºC with a flow rate of 0.8 mL min^{-1}using the following program: 0.01 min - 92% A, 8% B; 9.30 min - 83% A, 17% B; 10 min - 0% A, 90 % B, 10% C; 11 min - 0% A, 90% B, 10% C; 11.50 min - 92% A, 8% B; 14.50 min - 92% A, 8% B. Chromatograms were recorded at 250 nm. Standards of D-mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, D-glucose, D-

galactose, L-xylose, D-arabinose, and L-fucose (Sigma, Australia) were used to identify and quantify the derivatized monosaccharides.

Quick Start™ Bradford Protein Assay Kit (BioRad) was used to quantify water soluble protein concentrations. Five milligrams of freeze-dried mucus was diluted in 1 mL of water (1 mg of mucus in 200 uL of water). Bovine Serum Albumin with different concentrations were prepared to create a calibration curve. Each sample and standard were performed in triplicate to calculate the mean and standard deviation of protein concentration for each sample.

Microbial Gene Analysis

DNA was extracted from Sterivexes following Kerr et al., 2023 without modification using the Macherey-Nagel Tissue DNA extraction kit (Macherey-Nagel, Germany). DNA was quantified by a Quibt fluormeter (Invitrogen, USA). Samples were diluted in thin-walled PCR tubes to 2 ng/µL in 50 µL if possible. If the concentration of a sample was less than 2 ng/µL, 50 µL undiluted sample was used for sonication. Samples were sonicated using the QSonica Q800R model (QSonica Sonicators, USA). Samples were sonicated at 20% amplitude for 9 minutes with alternating sonication periods of 15 seconds of sonication followed by a 15 pause. Samples were sonicated at 4°C. Libraries were prepared using the xGen™ DNA Library Kit with an input fragment size of 350 base pairs according to the manufacturer instructions. Libraries were sequenced by the Australian Genome Research Facility on the Illumina Next Seq (300 base pairs) (Illumina, USA). Microbial functions were annotated using the atavide pipeline (https://github.com/linsalrob/atavidee) on the Flinders University HPC Deepthought (Flinders University 2021). Metagenomes are available on the Sequence Read Archive under BioProject PRJNA1245075.

The sequenced metagenomes were analyzed though an inhouse pipeline "Atavide" (Roach and Edwards 2021), which includes sequencing quality control using fastp (Chen et al., 2018) and host filtering using minimap2 (Li 2018) and SAMtools (Li et al., 2009). Gene functions are annotated using mmseqs2 (Steinegger and Soding 2017) against the UniRef databases (Suzek et al., 2007). Genes were annotated using the SEED Subsystems hierarchical structure that sorts annotated genes into broad metabolic processes Subsystems Level 1 being the broadest category and Subsystem Function being the most specific. We focused on only Subsystem Level 1 pathways carbohydrate metabolism and membrane transport. Within these are metabolic pathways (Subsystems Level 2 such as monosaccharides or carbon fixation). These are followed by more specific Level 3 Subsystems, such as fucose utilization, and finally a Function (sometimes including a gene name) similar to descriptions by (Dinsdale et al., 2008). For example, Alpha-glucosidase (Function) falls within the D-Galacturonate and D-Glucuronate Utilization (Subsystem Level 3) within the monosaccharide (Subsystem Level 2) and finally Carbohydrate (Subsystem Level 1) classifications.

Statistical Analysis

Monosaccharide and microbiome data was analyzed to determine differences mucus constituents and genes content between Elasmobranch species, and all statistics were conducted in RStudio version 4.4.2. All plots were created in RStudio using ggplot2 (Wickham et al., 2016).

Monosaccharide weight percent was calculated using the concentration of each monosaccharide relative to the sample input dry weight and sample volume over the total sample input mass. Shapiro and Levene's tests were used to determine normality and

homogeneity of variance. Assumptions were not met for all monosaccharides, so Kruskal Walis tests were used to compare individual monosaccharide abundances followed by Dunn's test. The vegan package was used to determine Bray-Curtis distances which were compared with the ADONIS and Pairwise ADONIS PERMANOVA function and visualized with an NMDS ordination (Oksanen et al., 2024, Martinez 2017). Mean weight percent and standard error were calculated for each monosaccharide within a host and visualized using grouped bar plot.

Microbial genes were standardized (relative abundance) for each subsystem level to account for varying library sizes. For example, the relative abundance of carbohydrate Subsystem Level 1 genes category was calculated by taking reads of each pathway within that category over the total carbohydrate genes in a sample. Manual search of all functional pathways resulted in the inclusion of one Membrane Transport pathway, utilization of glycans and polysaccharides (PULS), in gene analysis reported as presence absence. Ratio of photosynthesis genes to respiration genes was calculated by taking the reads in each of those pathways over the total reads in each metagenome.

Results

The mucus content on the seven species of sharks and rays was generally low and the mean water content was high (Table 4.1). Indeed, no mucus could be obtained from fiddler rays, seven-gilled sharks or the coffin ray and only one Port Jackson shark individual.,

Table 4.1: Elasmobranch sampling effort and final mucus sample count. The final samples include pooled samples from multiple individuals.

Host	Individuals Caught	Final samples	Mean water content in wet mass	Mean salt content in dry mass
Angelshark	12	3 (2 pool)	95.2 %	77.4 %
Coffin Ray	1	0	NA	NA

Eagle Ray	9	6 (1pool)	94.5 %	28.9 %
Fiddler Ray	21	0	94.5 %	82.3 %
Port Jackson Shark	15	1	90.0 %	50.0%
Sevengill shark	8	0	NA	NA
Whitespotted skate	18	6 (3 pool)	95.3 %	58.7%

Mucus Composition

We quantified the abundance of eight monosaccharides in four species of Elasmobranchs: (*Myliobatis tenuicaudatus*), Port Jackson sharks (*Heterodontus portusjacksoni*), Australian angelsharks (*Squatina australis*), and whitespotted skates (*Dentiraja cerva*) (Table 2). Overall, each species had a low abundance of carbohydrates in mucus between 0.08% and 9.16% w/w. The key monosaccharides were glucose, glucosamine, galactose and fucose as they were present in nearly all individuals tested (Figure 4.1). Across all host species tested, glucose made up the largest proportion of monosaccharides in mucus carbohydrates (between 0.11% and 3.44%) (Table 4.2).

Table 4.2: Relative abundance (% w/w) of monosaccharides (mean ± standard deviation). Pooled samples containing more than one individual have been included in calculations.

Monosaccharide	Angelshark	Eagle Ray	Whitespotted Skate	Port Jackson Shark
Arabinose	0	0	0.04 ± 0.08	0
Fucose	0.18 ± 0.14	0.26 ± 0.19	0.03 ± 0.07	0
Galactose	0.13 ± 0.34	0.58 ± 0.22	0.07 ± 0.09	0.70
Glucose	0.21 ± 0.16	1.89 ± 0.90	0.18 ± 0.18	1.65
Glucuronic Acid	0	0.08 ± 0.08	0	0
Glucosamine	0.28 ± 0.34	2.22 ± 0.94	0.07 ± 0.15	0.39
Mannose	0	0.07 ± 0.03	0	0.60
Ribose	0	0.17 ± 0.06	0	0.10
Total Carbohydrate	0.81 ± 0.75	4.26 ± 2.69	1.68 ± 2.11	2.90

Table 4.3: Comparisons of monosaccharide abundance across host species.

Monosaccharide	Kruskal Walis chi-squared	df	p-value
Arabinose	5.1734	3	0.07527
Fucose	2.4534	3	0.4838
Galactose	5. 4412	3	0.1422
Glucose	7.9632	3	0.04678
Glucuronic Acid	5.1734	3	0.07527
Glucosamine	9.6549	3	0.02174
Mannose	9.8125	3	0.02023
Ribose	10.91	3	0.0122

Eagle rays had the highest proportion of carbohydrates in mucus ranging from 3.43% to 9. 16% of the biomass dry weight (Figure 4.1). Whitespotted skate mucus had the highest proportion of fucose, eagle rays had the highest proportion of glucosamine (Figure 4.1). Arabinose was only detected in skate mucus, while ribose was only detected in eagle ray mucus.

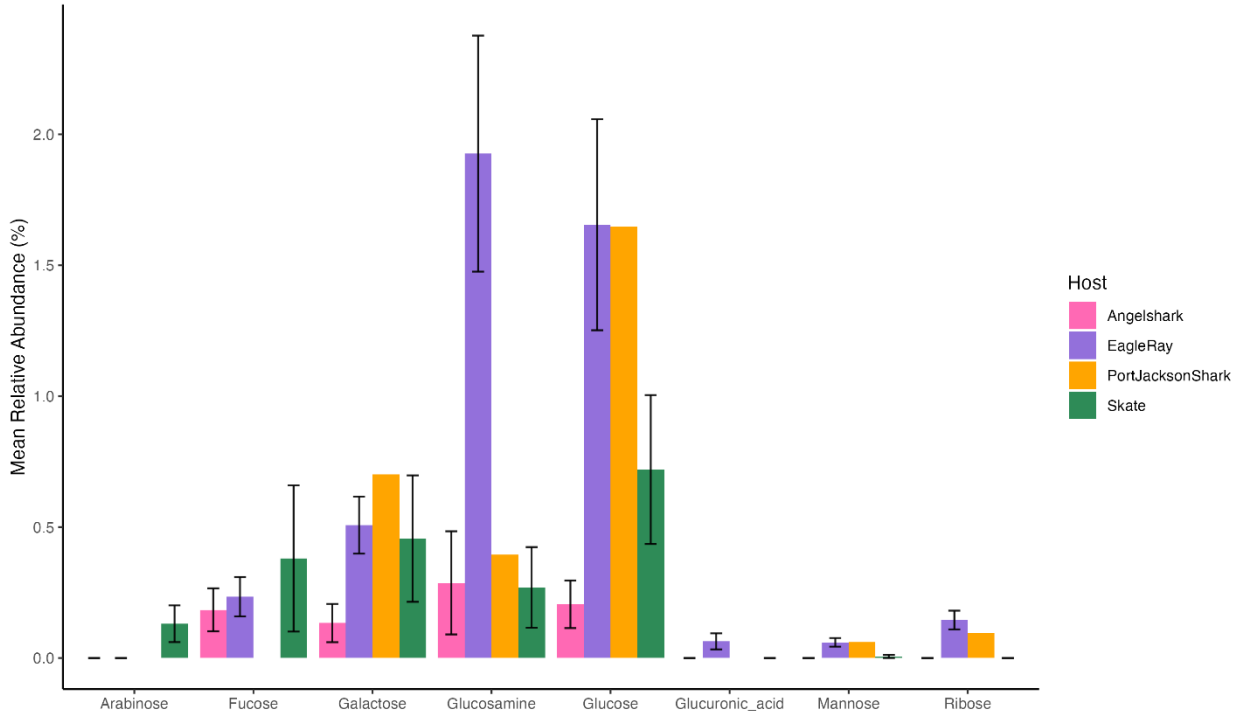

Figure 4.1: Average relative abundance (% w/w) of monosaccharides in each host. Error bars indicate standard error except for Port Jackson sharks where n = 1.

Mucus composition was compared between skates, eagle rays, and angelsharks (including pooled samples). Eagle ray and skate mucus composition were significantly different (PERMANOVA p-adj = 0.027, F = 5.8, df = 1). No differences were observed between angelsharks and either skates or eagle rays (PERMANOVA p-adj > 0.05 for both comparisons). Despite the lack of statistical significance, eagle rays appear to cluster close together, especially the individual mucus samples (Figure 4.2). The abundance of glucosamine ribose and mannose differed significantly between hosts (Table 4.3). Glucosamine and ribose were significantly different between eagle ray and skate mucus (Supplementary Table 4.2). Mannose and ribose were significantly different between angelshark and eagle ray mucus (Supplementary Table 4.2).

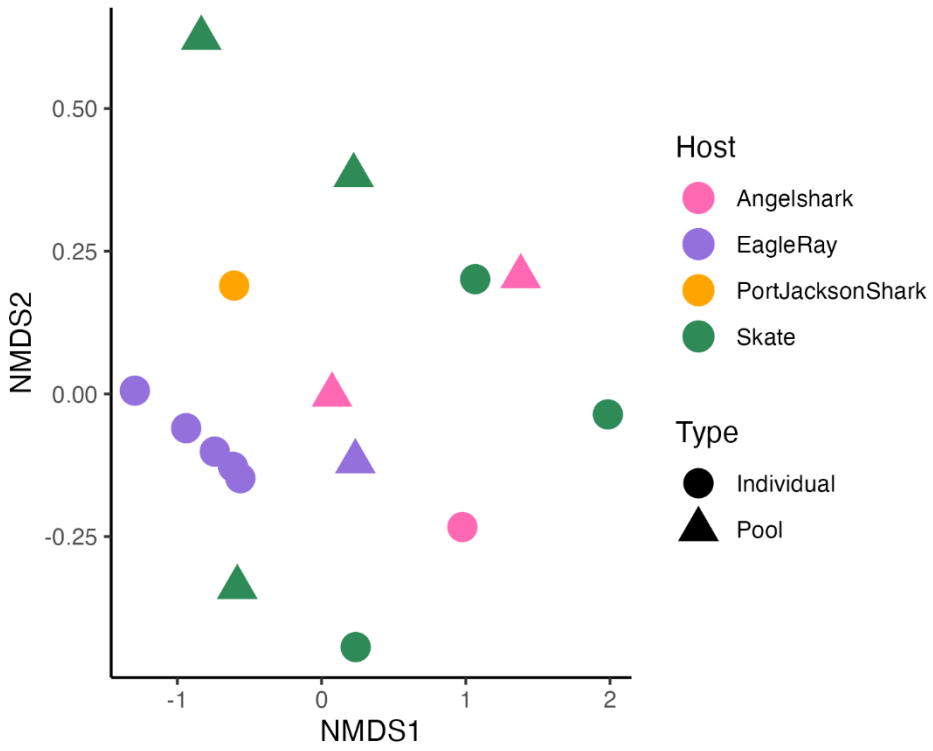

Figure 4.2: The monosaccharide components of the eagle ray were consistent across individuals whereas the skate mucus showed greater variability. Similarity was calculated using a non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities of the monosaccharide components, showing both pooled and individual samples.

The concentration of soluble proteins in epidermal mucus in eagle rays ranged from 19.05 to 50.24 ug/mg, with an overall mean of 36.48 ug/mg. The single Port Jackson sample has a concentration of 16.76 ug/mg (Table 4.4).

Table 4.4: Bradford assay protein concentrations in ug/mg.

	EagleRay129	EagleRay131	EagleRayGSV303	PJGSV402
Average (ug/mg)	50.24	40.16	19.05	16.76
Standard Deviation	2.59	2.05	2.25	1.39

Microbial Genes

The epidermal microbial community from 12 Australian angelsharks, 9 eagle rays, 15 Port Jackson sharks, 18 whitespotted skates, and 20 water samples were compared. The ratio between photosynthesis genes to respiration genes was on average was 1:5 in the water column and 1:19 in the host microbiomes, suggesting the water column microbes were producing carbon while the host microbes were utilizing carbon. While the Carbohydrate metabolism (Subsystem Level 1) was found in all metagenomes, the water metagenomes had a higher mean relative abundance of carbon fixation (a Subsystem Level 2 pathway) (30.4%) compared with the host-associated metagenomes (25.0% carbon fixation). Eagle rays showed the lowest proportion of carbon fixation genes among the host-associated metagenomes (20.0%) suggesting readily available carbon sources on the skin of the rays compared with the water column. Host metagenomes had a higher average relative abundance of monosaccharide (14.3%), di- and oligosaccharide (16.0%), and polysaccharide (11.6%) than water column metagenomes (11.6%, 14.1%, and 5.7% respectively) (Figure 4.3).

We focused on the distribution of the subsystems within in the three saccharide groups across the host and water metagenomes (Figure 4.4). Microbiomes from all host organisms had genes to utilize sugars present in the mucus. D-Galacturonate and D-Glucuronate utilization (the acidic form of galactose and glucose) pathway has a high relative abundance across all hosts (angelshark 8.3%, eagle ray 7.3%, Port Jackson sharks 5.8% and skate 8.2%), and was lower in water microbiomes. The galactose utilization pathway was made up 12-14% of carbohydrate genes (Figure 4.5). Lactose (a disaccharide made up of glucose and galactose) utilization has a relative abundance of about 3% in host-

associated metagenomes (Figure 4.4). Glycogen metabolism has a relative abundance of between 10 and 13% across the sharks and rays but was only 5% in the water column where glucose molecules are rare (Figure 4.4). L-fucose utilization is highest in eagle rays (15.0%) compared to angelsharks (1.6%) and skates (1.2%). D-Glucosamine utilization was absent in all microbiomes except one angelshark metagenome, where the relative abundance is negligible (less than 0.001%), suggesting that the glucosamine which is part of the core of the glycans is not being used directly by the microbes. However, several transporters, such as TonB and phosotransferases, could transfer GlcNAc into the cell. Arabinose utilization pathway was less than 1% of carbohydrate genes and a negligible abundance of genes in the metagenome overall, consistent with the lack of arabinose in the mucus. No genes for xylose or rhamnose utilization were identified in any Elasmobranch metagenome.

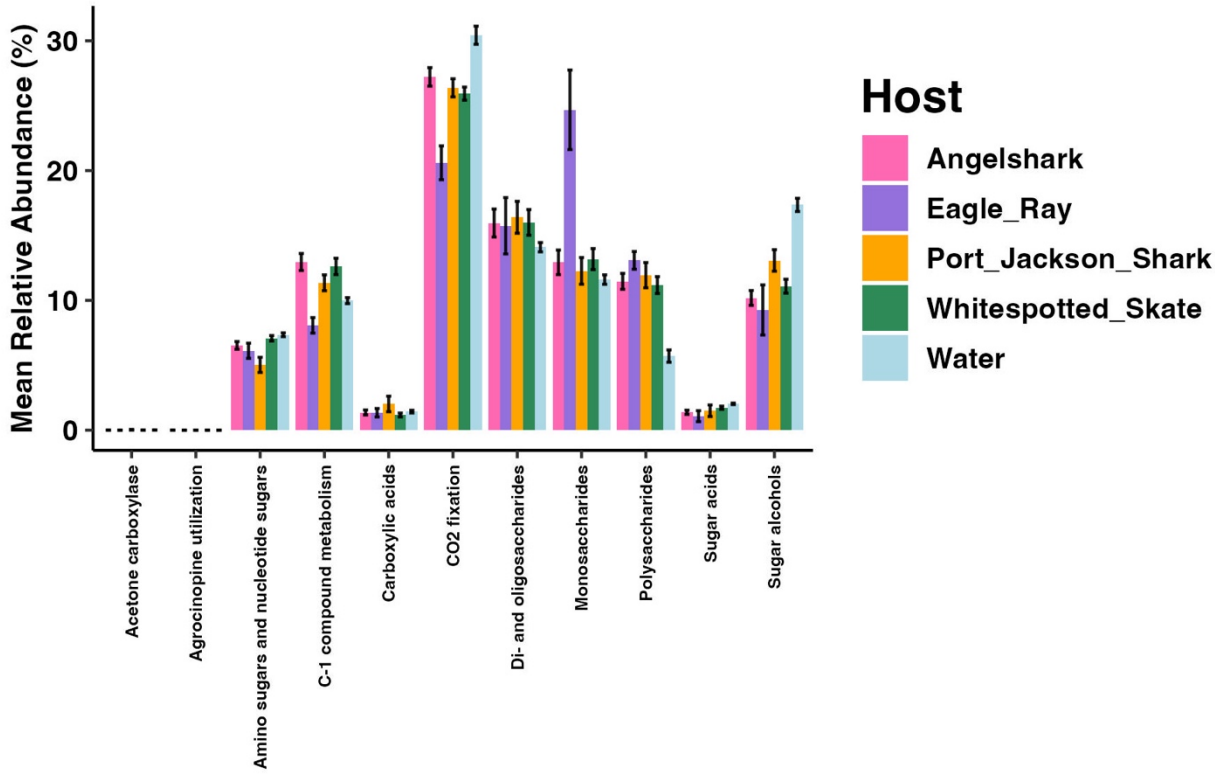

Figure 4.3: Distribution of Subsystem Level 2 genes that fall within carbohydrate metabolism (Subsystem Level 1) in host and water column metagenomes.

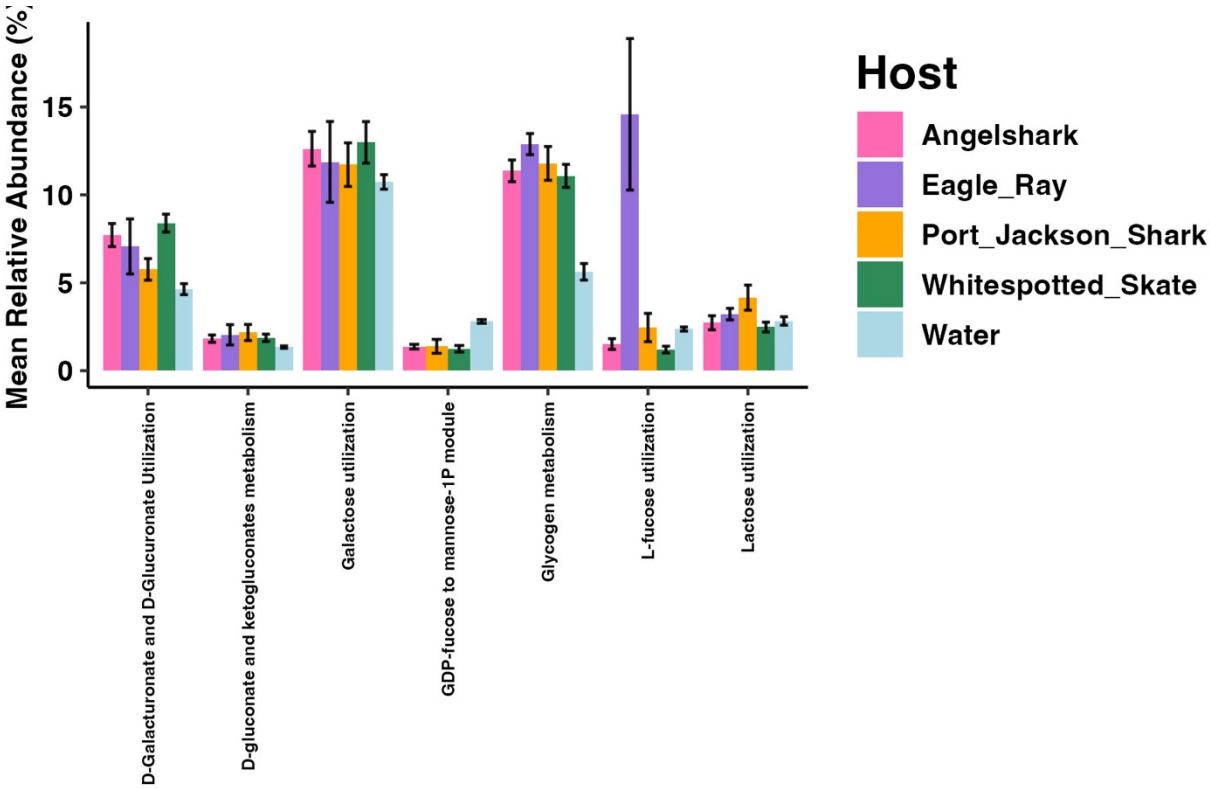

Figure 4.4: Abundant (greater than 1%) microbial functional genes (Subsystem Level 3) within the monosaccharide, di and oligosaccharide, and polysaccharide Subsystem Level 2 pathways from the shark and ray host microbiomes compared with the water column microbiomes.

The relative abundance of glycogen metabolism in the sharks and ray microbiomes was double that in the water column microbiomes, reflecting the availability of the glycans in the mucus. The proportion of glycogen metabolism pathways was higher in the sharks and rays compared with the water column, in particular 4-alpha-glucanotransferase (amylomaltase) (EC 2.4.1.25) and glycogen phosphorylase (EC 2.4.1.1) were in highest abundance on the skin of eagle rays (Table 4.4). In contrast, the glycan synthesis pathways were more abundant in the water column, particularly ADP-glucose transglucosylase (EC 2.4.1.21) (Table 4.4) a gene in the pathway to build carbohydrates, suggesting that a greater proportion of genes are available for the synthesis of glucose.

The utilization systems for glycans and polysaccharides (Subsystem Level 3) pathway belongs to the membrane transport (Subsystem Level 1) classification. This group of genes was present in host and water column metagenomes in similar proportions. Four genes in the Sus operon (SusA, D, C, and R) are present in host metagenomes.

Table 4.4: Average relative abundance (%) of genes within glycogen metabolism (Subsystem Level 3) across the fours Elasmobranch and water column microbiomes. Pathways are grouped into Utilization (breaking down glycogen) and Synthesis (building glycan molecules).

Glycogen Metabolism		Angelshark	Whitespotted Skate	Eagle Ray	Port Jackson Shark	Water
Glycan Utilization	4-alpha-glucanotransferase (amylomaltase) (EC 2.4.1.25)	13.85	10.58	14.61	14.92	9.85
	Alpha-glucosidase, family 31 of glycosyl hydrolases, COG1501	0.68	1.04	2.48	0.17	1.73
	Glycogen phosphorylase (EC 2.4.1.1)	30.84	30.14	36.86	29.19	25.52
	Limit dextrin alpha-1,6-maltotetraose-hydrolase (EC 3.2.1.196)	16.77	20.09	11.06	19.87	15.26
	Maltodextrin phosphorylase (EC 2.4.1.1)	0.00	0.00	0.00	0.00	0.01
	Putative glycogen debranching enzyme, archaeal type, TIGR01561	0.91	0.86	0.12	0.12	0.77
Glycan Synthesis	1,4-alpha-glucan (glycogen) branching enzyme,	18.97	18.88	13.17	19.24	17.45

GH-13-type (EC 2.4.1.18)					
Glucose-1-phosphate adenylyltransferase (EC 2.7.7.27)	4.07	2.94	6.35	2.42	4.26
Glycogen branching enzyme, GH-57-type, archaeal (EC 2.4.1.18)	0.23	0.57	0.12	1.27	3.79
Glycogen synthase, ADP-glucose transglucosylase (EC 2.4.1.21)	13.65	14.84	15.16	12.50	21.01
Predicted glycogen synthase, ADP-glucose transglucosylase (EC 2.4.1.21), Actinobacterial type	0.03	0.05	0.06	0.28	0.36

Discussion

We described the Elasmobranch epidermal metabolic potential and mucus carbohydrates and provided the first description of the interactions between the microbes and Elasmobranch hosts. We identified that Elasmobranchs mucus had a low proportion of carbohydrates and were dominated by four monosaccharides (glucose, galactose, glucosamine and fucose). The associated microbial community had genes to detect the presence of the monosaccharides, transport them into the cell and utilize them as carbon sources. Therefore, epidermal microbiomes are adapted to life on the surface of the sharks and rays where they consume carbon rather than produce carbon via photosynthesis unlike the microbes in the surrounding water column where photosynthesis dominates.

Carbohydrates comprised less than 10 % of the total mucus biomass of all Elasmobranch samples (Figure 4.1), in distinct contrast to coral mucus where carbohydrates comprise up 80 % of the mucus (Wild et al., 2010). In sharks and rays, the diversity of monosaccharides and their relative abundance varies from species to species, but several key monosaccharides are abundant across clades. Eagle rays had seven types of monosaccharides, the two most abundant groups being glucose and glucosamine. In contrast, angelsharks had the fewest unique monosaccharides (4) out of those tested. In corals, arabinose and mannose are common (Thobor et al., 2024), whereas those monosaccharides were absent in Elasmobranch mucus. Instead, Elasmobranch mucus was dominated by glucosamine, glucose, galactose and fructose. The relative abundances of monosaccharides were distinct across Elasmobranch species similar to the variability of mucus constituents across coral clades (Thobor et al., 2024). The mucus constituents of the eagle rays were consistent, even though one sample was collected a year later than the others, suggesting that the mucus composition of rays is relatively stable. The mucus from the skates had higher variability in monosaccharide constitutes, even though all individuals were collected at a similar location and time. Fish epidermal mucus fluctuated with changes in environmental conditions (Fernández-Alacid et al., 2018) suggesting that the mucus environment is dynamic. Therefore, further research is required to understand the extent to which saccharide concentration in the mucus on Elasmobranchs varies with environmental conditions.

Of the monosaccharides present, galactose, glucosamine and glucose were present across all hosts and were highest in abundance (Figure 4.2). Shark and fish mucus was rich in glycoproteins made up of these monosaccharides (Bachar-Wikstrom et al 2023a, Jin et al., 2015). These three monosaccharides are the building blocks of the eight core glycan

structures identified in all animals, to which other monosaccharides are added to produce a diversity of O-glycans (Watson et al., 2015). Galactose is attached to the base N-acetyl galactosamine in Core 1 and 2 glycan structures, the most common of the eight core glycans (Watson et al., 2015). Core 1 and 2 glycans are abundant in Elasmobranchs, including dogfish, little skate and chain catshark skin mucus, and in Osteichthyes fish including arctic charr, Atlantic salmon intestinal mucus (Venkatakrishnan et al., 2019, Jin et al., 2015, Bachar-Wikstrom et al., 2023a). Our results show that monosaccharides that make up key glycans occurred in the epidermal mucus of the four Elasmobranch species investigated. In contrast, galactose was either not present or made up a small fraction of total carbohydrates in coral mucus (Hadaidi et al., 2019; Thobor et al., 2024). The presence and abundance of galactose in all Elasmobranchs measured to date suggests that core 1 and 2 glycans are likely to be constituents of the mucus and important in forming mucus oligosaccharides, but the biological role of galactose-containing glycoproteins is unknown.

N-Acetylgalactosamine residues are attached to serine or threonine amino acid to form Core glycan structures and N-Acetylglucosamine (GlcNAc) is added to form Core 2, 3, 4 and 6 O-glycans (Watson et al., 2015). Glucosamine was present in all sharks and rays, similar to five species of coral suggesting it is a key mucus monosaccharide conserved across clades (Hadaidi et al., 2018). N-acetylglucosamine acts as a protection mechanism for the hosts by inhibiting the biofilm formation of *E. coli*, under laboratory conditions (Sicard et al., 2018). Motile strains of *Vibrio,* a common bacteria associated with sharks and rays (Kerr et al., 2023; Doane et al 2020), metabolize the terminal N-acetylglucosamine to invade the host immune system, whereas strains unable to metabolize N-acetylglucosamine are non-motile and have reduced pathogenicity (Fekete & Buret 2023).

Monosaccharide genes in the microbiome including galacturonate and glucuronate utilization genes were of considerable relative abundance across all host species microbiomes, especially eagle rays (Figure 4.4). The most abundant function within the galacturonate and glucuronate pathway was Alpha-glucosidase. Alpha-glucosidase breaks down polysaccharides by cleaving alpha 1-4 glycosidic bonds releasing free glucose (Fox 2017), suggesting that the commensal bacteria are breaking down complex sugars, to use the carbon as an energy source.

Within the di- and oligosaccharide utilization subsystem, 17 genes for Galactose metabolism were present (Figure 4.4). UDP-glucose 4-epimerase, alpha galactosidase, and sodium-dependent galactose transporter were most abundant within the galactose metabolism subsystem. UDP-glucose 4-epimerase is an enzyme that metabolizes galactose into glucose (Wilson & Hogness 1964). In human infant gut microbiomes *Bifidobacterium longum* efficiently metabolizes O-glycans in human milk oligosaccharides and mucin glycans (Nam et al., 2019). Alpha galactosidase are enzymes capable of cleaving a galactose residue from a longer oligosaccharide molecule (Zhao et al., 2018). Sodium glucose transporters regulate glucose uptake into a cell (Poulsen et al., 2015). Epidermal microbiomes had a high relative abundance of enzymes to break down complex oligosaccharides and take up monosaccharides from their environment which indicated bacteria have the genotype for metabolizing sugars found in Elasmobranch mucus.

Whitespotted skate mucus had a high concentration of fucose, consistent with little skate mucus glycans (Bachar-Wikstrom et al., 2023a). In addition, proteins involved in fucosylation (including GDP-L-fucose synthase) were identified on the skin of Elasmobranchs (Bachar-Wikstrom et al., 2023b). Similarly, we identified a high relative

abundance of fucose utilization genes in the metagenomes (Figure 4.4), supporting the hypothesis by Bachar-Wilkstrom et al (2023b) that fucosylation of proteins in shark mucus regulates host-microbiome communication and immunity. Eagle rays had an especially high relative abundance of L-fucose utilization genes (15% average relative abundance) suggesting the commensal bacteria utilize fucose within the mucus layer as an energy source. Therefore, the high mucus turnover rates on stingrays may reduce the abundance of microbe and cause increased intra-individual variation in microbial diversity compared with sharks (Burgess et al., 2018; Kerr et al., 2023)

Glycogen metabolism was also overrepresented in Elasmobranch microbiomes compared to water column metagenomes (Table 4). Within that subsystem, glycogen phosphorylase, and alpha-1,6-maltotetraose-hydrolase had high relative abundance in Elasmobranch microbiomes. Glycogen phosphorylase breaks polymers to release glucose which can be used for energy (Migocka-Patrzałek & Elias 2021). Alpha-1,6-maltotetraose-hydrolase is a “de-branching enzyme” that hydrolyzes alpha1-6 glycosidic linkages to break down glycogens (Jeanningros et al., 1976). Glycogen metabolism genes are over-represented in shark microbiomes compared to the water column and therefore could be breaking down host mucus if these genes are expressed. In wild leopard shark epidermal microbiomes, there was a high relative abundance of carbohydrate metabolism genes and 15 of the 17 most abundant bacteria carried glycolysis pathway genes (Doane et al., 2022). In the epidermal microbiome of two stingrays, *Urobatis halleri* and *Myliobatis californica*, functional analysis identified a high proportion of genes associated with carbohydrate metabolism and low abundance of photosynthetic genes. The metagenome assembled genomes (MAGs), including novel *Alcanivoracaceae*, Gammaproteobacteria, *Rhodobacteraceae* and

Moraxellaceae species, from these hosts and all had genes for the utilization of monosaccharides, di- and oligosaccharides, but sugar alcohols genes were less abundant (Kerr et al., 2023). In contrast, utilization of more complex polysaccharides was present in high abundance in 4 of the 5 bacterial species excluding *Rhodobacteraceae*. In addition, the relative abundance of carbohydrate synthesis genes was higher in captive compared to wild leopard sharks. Genes coding for enzymes involved in the breakdown of these saccharides i.e., beta-glucosidase, were correspondingly higher in captive shark epidermal metagenomes compared to semi-captive and wild counterparts (Goodman et al., 2023), suggesting that in captivity the mucus production may increase on the sharks due to lower swimming speeds or higher nutrient availability.

In addition to specific enzymes needed to break down mucus carbohydrates, bacteria also need to import those carbohydrates into the cell (Ouwerkerk et al., 2013). Utilization systems for glycans and polysaccharides (PULs, SUS) fall within the Subsystem Level 2 category membrane transport classification (Overbeek et al., 2014). The Sus operon in Bacteroides contains eight genes (SusRABCDEFG) which facilitate the recognition, binding, and transport of carbohydrates into the bacterial cell (Brown & Koropatkin 2021, Foley et al., 2016; Tuson et al., 2018). Four Sus genes (R, A, C, and D) are represented in all Elasmobranch epidermal microbiomes. These genes have been well described in the *Bacteroides* genus, which has polysaccharide utilization loci that metabolize host O-glycans, (Koropatkin et al., 2012) and *Lactobacillus* that binds efficiently to mucus glycans (Van tassell & Miller 2011). The presence of SusR (recognition of glycans) and SusD (binding of glycans) suggests that bacteria from shark and ray epidermis sense and bind to mucus oligosaccharides.

Transporter genes including Ton and Tol transport systems occur in Elasmobranch microbiomes in high relative abundance (Doane et al., 2022, 2023; Kerr et al., 2023). All MAGs from *U. halleri* and *M. californica* contained a high abundance of sugar transport genes (Kerr et al 2023). Shark and ray microbiomes contained necessary glycosidases (alpha-glucosidase and galactosidase) and de-branching enzymes which complete the Sus pathways suggesting they utilize glycans directly (Foley et al., 2016). Mucins in shark skin have been suggested to provide nutrients for beneficial microbes (Bachar-Wikstrom et al 2023a) and our metagenomic data provides evidence that shark-associated microbes possess the genetic machinery for processing complex carbohydrates. Metagenomes cannot demonstrate that these genes are expressed, but a close relationship exists between gene presence and expression (Franzosa et al., 2014). However, further transcriptomic or culture-based experiments are required confirm these relationships. We provide the first conceptual model of the interactions between the host mucus and the epidermal microbiomes from the Elasmobranch epidermis (Figure 4.5).

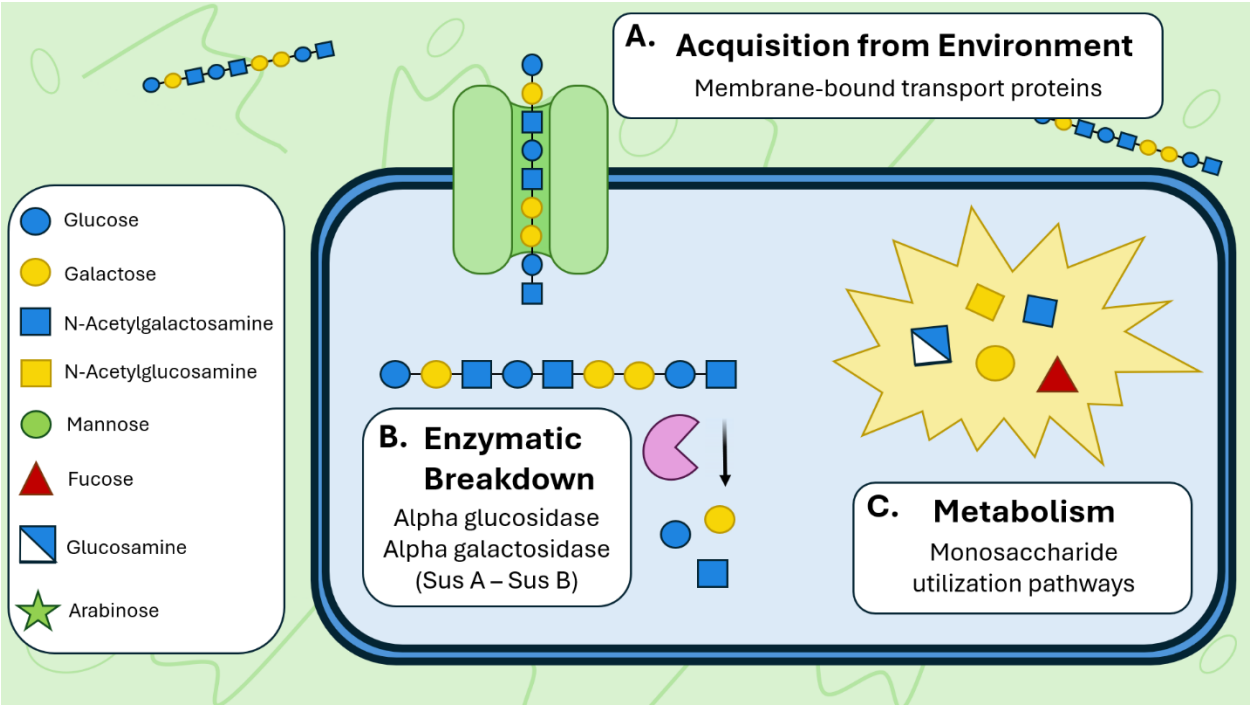

Figure 4.5: Conceptual diagram of potential bacterial metabolism of sugars. A: Transport systems for importing oligosaccharides into the bacterial cell. B. Enzymes for breaking down polysaccharides into monomers for energy use. C: indicates utilization systems for monosaccharides present in shark mucus.

Caveats

The low amount of mucus on many Elasmobranchs makes sample collection is a major obstacle to the description of mucus content on fish and sharks. Elasmobranch mucus is highly elastic and is difficult to remove from the epidermal surface. Low sample volume is a significant obstacle to describing the mucus components of fish (Fernandez Cunha et al., 2024) and we show here for Elasmobranchs. The tissue method (absorbing surface mucus using a tissue) yields about 1 mL of mucus from sharks, and when pooled across multiple individuals was successfully utilized for protein and glycan identification (Bachar-Wikstrom et al., 2023a). However, we aimed to compare the mucus to the microbial genes directly, thus keeping the mucus from individuals separate was important. We attempted to collect of mucus from seven species and 84 individuals and found that for many organisms the mucus

did not absorb onto tissues, potentially because of the mucus composition or the position of the mucus under the dermal denticles. Sharks and rays posed an additional challenge with high concentrations of salt in the mucus (up to 93% of the dry mass before rinsing) which needs to be removed for HPLC analysis, further increasing the quantity required. The second major method for obtaining mucus from fish is scraping with a tool across the skin, while this method typically yields a higher concentration of proteins from fish mucus (Fernandez Cunha et al., 2024), it causes injuries and contaminates the sample with blood. Fortunately, unlike ray-fined fish, shark skin is more difficult to damage when scraping reducing contamination by blood (Kerr personal observations), but the dermal denticle inhibit contact of the tool with the mucus, resulting in low volume of mucus obtained. We were able to prioritize sampling individuals which resulted in relatively low sample size, and as such our results offer a preliminary analysis of monosaccharide concentration of Elasmobranch mucus.

Conclusions

Here we describe for the first time the monosaccharide composition of mucus from four Elasmobranch hosts and pinpointed the genes contained by the commensal bacteria to utilize the mucus as a carbon sources. We found four key monosaccharides across species (glucose, galactose, glucosamine, and fucose) which are components of mucus glycans in other Elasmobranch species (Bachar-Wikstrom et al., 2023a). Mucus composition within eagle rays was consistent across individuals collected a year apart. Each Elasmobranch species had different monosaccharide components in their mucus, with a significant difference between eagle rays and whitespotted skates. Microbial genes coding for carbohydrate pathways were generally more abundant in host microbiomes than water

column microbiomes. Overall, Elasmobranch microbiomes had a higher concentration of carbon utilization genes, compared with the water column microbiome and a lower concentration of photosynthetic genes suggesting the mucus is an important nutrient source for these commensal microbes. The Elasmobranch epidermal microbes possess the genetic machinery required for detecting, transporting, and metabolizing monosaccharides and other carbohydrates present in the host mucus suggesting the mucus is a selective mechanism for the microbiome.

DRAFT GENOMES OF FIVE BACTERIA ISOLATED FROM *CARCHARODON CARCHARIAS* (WHITE SHARK) AND *CARCHARHINUS BRACHYURUS* (BRONZE WHALER SHARK)

Context

White sharks are a charismatic yet highly controversial species in Australia. They are responsible for a large proportion of human-shark interactions, yet they also play an important role in maintaining prey populations in marine ecosystems. In South Australia, they are a protected species, and in many parts of the world their populations are declining due to overfishing (bycatch and culling), biomagnification, and climate change. During my candidature I worked on a collaborative review "Cull, Conserve Compromise – the White Shark Conundrum" which discusses the nuances of shark management to balance conservation needs and human safety (Butcher et al., in review; Appendix 2). This collaboration was an opportunity to explore white shark microbiomes, particularly *Vibrio* bacteria. *Photobacterium damselae* is an important shark gut symbiont (demonstrated in Chapter 1). In this chapter, I cultured and sequenced shark-associated isolates to expand what is known about white shark gut and skin bacteria, with the goal of culturing *P. damselae*. Although culturing *P. damselae* was unsuccessful, I successfully cultured and sequenced five isolates. This chapter is published in *Microbial Resource Announcements*.

Abstract

Here we isolated five bacterial species from the skin and cloaca of *Carcharodon carcharias* (white shark) and *Carcharhinus brachyurus* (bronze whaler or copper shark).

Announcement

Shark microbiology originated in response to shark bites (Buck et al., 1984). However, microbes are integral in shark health and ecology (Doane et al., 2020; Perry et al., 2021). Elasmobranchs support biodiversity in marine ecosystems but are globally threated by overfishing and biomagnification of heavy metals (Henderson et al., 2024; Weijs et al., 2015; Wosnick et al., 2021). Biomagnification is reflected in shark microbial communities where heavy metal resistance genes are enriched (Doane et al., 2017, 2022; Kerr et al., 2023; Hesse et al., 2022). We sequenced the genomes of bacterial isolates from *Carcharodon carcharias* (white shark) and *Carcharhinus brachyurus* (bronze whaler shark).

Sharks were caught on SMART drumlines at Port Macquarie, New South Wales (NSW DPI scientific (Ref. P01/0059(A)), Marine Parks (Ref. P16/0145–1.1) and Animal Care and Ethics (ACEC Ref. 07/08)) (Tate et al., 2021; Butcher et al., 2023). Swabs were collected from the skin and cloaca, placed in a sterile seawater-filled microfuge tube and transported on ice. The bacterial slurry (50 uL) was plated on Thiosulfate–citrate–bile salts–sucrose agar (Bacto Laboratories, Australia) and incubated at 18 °C until colonies formed. Colonies were isolated and picked for DNA extraction using Nucleospin Tissue Kit (Macherey-Nagel, Germany). DNA concentrations were quantified with a Qubit 4 fluorometer (Invitrogen, USA). Libraries were prepared with raw DNA without size selection using the Nanopore Rapid Barcoding Kit and sequenced on the MinION (flow cell R9.4.1) (Oxford Nanopore, United Kingdom). Default parameters were used for all bioinformatics tools. Guppy basecalling (V 6.5.7) converted raw signal to base pairs (Oxford Nanopore Technology). Hybracter (V 0.10.1) quality-controlled sequences and assembled contigs (Bouras et al., 2024). Average coverage is the mean coverage of all contigs in a genome. Taxonomic

classification was determined using a BLASTN search of the entire genome sequence (Altschup et al., 1990), and Bakta (V 1.10) for functional annotation (Schwengers et al., 2021). Bandage (v0.8.1) was used to visualize genomes (Wick et al., 2015). Sequences are available under BioProject PRJNA1107358 (Table 1). Annotations are available on FigShare (https://figshare.com/articles/dataset/Bakta_annotations_for_bacteria_isolated_from_white_and_bronze_whaler_sharks/28702667).

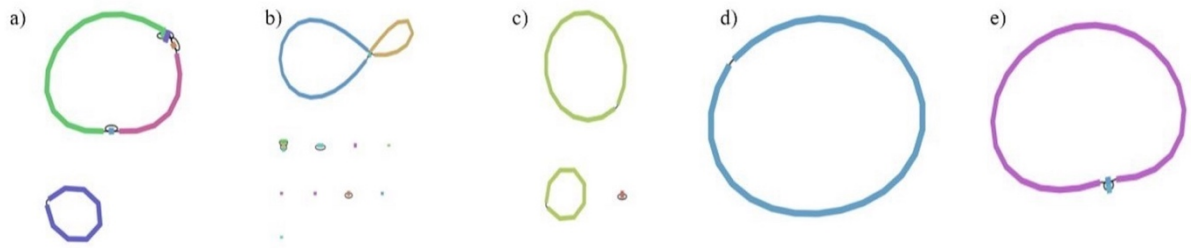

Figure 5.1: Visualization of genome assemblies. a. *Vibrio toranzoniae* strain ENK1 b. *Aliivibrio fischeri ES114* strain ENK5 c. *Vibrio atlanticus* strain ENK4 d. *Shewanella sp. WPAGA9* strain ENK2 e. *Shewanella woodyi* strain ENK6.

Genomes were complete and contained 4,750,424 to 5,949,190 base pairs in 1-11 contigs (Fig. 1, Table 1). Antimicrobial and heavy metal resistance genes were present in all genomes. Cloaca isolates have class A beta-lactamase, heavy metal efflux pump, trimethylamine-N-oxide (TMAO) reductase, and urease genes which are lacking from skin isolates. TMAO and urea are used to maintain osmotic balance by Elasmobranchs (Forster & Goldstein 1976). Presence of these genes may indicate compounds utilized by shark microbes. Cation Diffusion Facilitator (CDF) family efflux transporter DmeF gene was

present in skin isolates and may provide resistance to heavy metals (Kolaj-Robin et al., 2015).

Table 5.1: Isolate genome statistics.

Species	Host	Genome length (bp)	Number of reads	Sequence N50	Contigs	Assembly N50	GC content (%)	Average Coverage	Annotated genes	SRA GenBank
Vibrio toranzoniae strain ENK1	*C. carcharias* cloaca	4,962,167	294,978	7957	3	1,510,937	43.92	23.3	4,906	SRS21193063 JBLPGB000000000.1
Aliivibrio fischeri ES114 strain ENK5	*C. carcharias* cloaca	4,750,424	735,427	8892	11	2,972,306	38.42	52.4	4,982	SRS21193066 JBLOOV000000000.1
Vibrio atlanticus strain ENK4	*C. carcharias* skin	4,983,971	1,060,052	6743	3	3,372,530	44.07	98.3	4,709	SRS21193065 CP171407.1 CP171408.1
Shewanella sp. WPAGA9 strain ENK2	*C. carcharias* skin	4,871,077	342,618	8310	1	4,871,077	40.83	29	4,537	CP171409.1 SRS21193064 CP171406.1
Shewanella woodyi strain ENK6	*C. brachyurus* cloaca	5,949,190	1,352,592	6366	1	5,949,190	43.43	84	5,784	SRS21193067 CP171410.1

CHAPTER 6: PHYLOSYMBIOSIS IS MAINTAINED ACROSS GLOBAL BENTHIC ELASMOBRANCH MICROBIOMES

Context

Previous chapters have explored microbiome diversity in different contexts to explore host and environmental drivers of microbial communities in hosts sampled in the same environment or very close together (300 km or less). Here I compare Elasmobranch skin microbiomes across oceans by comparing the sequence similarity and Metagenome Assembled Genomes from hosts sampled in southern California and South Australia. I use a database independent approach to assess the influence of hosts that are phylogenetically similar but live across the globe (phylosymbiosis). I use short read (150 & 350 base pair) data from my previous chapters I performed co-assemblies for each host and compared sequences, contigs and MAGs to investigate similarities between hosts of the same genus and hosts from the same country. I aimed to identify genus-specific MAGs and location specific MAGs to help better understand which microbes in the community are host and which are environment specific. This chapter utilizes metagenomic data from several projects, maximizing existing sequence data for understanding host-microbe ecology in sharks, rays, and skates. This work is being prepared for publication in the International Society for Microbial Ecology Journal.

Abstract

Sharks and rays are ancient vertebrates that evolved over 400 million years ago. They are critical to coastal ecosystem health but are particularly susceptible to overfishing, pollution, and climate change. Their importance to the ecosystem and evolutionary success make them key species for identifying eco-evolutionary patterns. Here we take a metagenomic approach to analyze how host (phylosymbiosis) and location (California vs South Australia) impact the microbial communities of fourteen species of Elasmobranchs

and the water column. We find that metagenomes are more similar within a host order than across host order, or when compared to the water column. The Myliobatiformes order showed high sequence similarity, specifically the *Myliobatis* genus (represented by two species, one from each location) are more influenced by host than location. Metagenome Assembled Genome (MAG) functional genes were significantly influenced by host but not location. Resistance (antimicrobial and heavy metal) genes were not influenced by host or location. Overall, our results show host phylogeny (phylosymbiosis) is the primary driver of microbiome similarity and MAG functional genes.

Introduction

Phylosymbiosis is the eco-evolutionary pattern in which closely related hosts have more similar microbiomes than more distantly related hosts (Lim and Bordenstein 2020). Phylosymbiosis occurs in hosts from sponges to mammals, driven by microbe-microbe and microbe-host interactions (Mallott & Amato 2021). In marine systems, phylosymbiosis is observed in marine invertebrates (O'Brien et al., 2020), corals (Pollock et al., 2018), fish (Chiarello et al., 2018), sharks (Doane et al., 2020), and mammals (Apprill et al., 2020) to varying degrees. Some hosts like fish, the relationship between phylogeny and microbiomes is weak or absent (Chiarello et al., 2018; Doane et al., 2020). Host phylogeny can affect microbiomes at some body sites more than others, for example coral mucus is more impacted by environmental factors, while skeleton and tissue were more affected by host phylogeny (Pollock et al., 2018).

Elasmobranchs (sharks, rays and skates) evolved over 400 million years ago (Aschliman et al., 2012; Villalobos-Segura et al., 2022) and represent ancient vertebrates. Elasmobranch genomes are large and have remained remarkably stable over millions of

years (Marra et al., 2018; Sáez et al., 2024). They represent an early vertebrate form, sharing traits like viviparous birth with mammals which could contribute to phylosymbiosis which are absent in other fish species (Mallott & Amato 2021). Rays and skates though, share traits with fish like prominent epidermal mucus (Meyer & Seegers 2012), which could complicate the phylogenetic signal seen in other pelagic shark epidermal microbiomes (Doane et al., 2020). Stingray microbiomes are more impacted by the surrounding environment than sharks when moved from the wild to captivity (Goodman et al., 2022; Pinnell et al., 2021) indicating a higher degree of environmental influence than shark microbiomes. Rays also have high variation in their epidermal microbiomes (Clavere-Graciette et al., 2023; Kerr et al 2023) unlike sharks which maintain low intraspecific variation (Doane et al., 2017, 2020; Goodman et al., 2023). The unique morphological and ecological traits of demersal Elasmobranchs serve as an interesting system for exploring microbe host interactions.

Coastal temperate ecosystems are incredibly productive hotspots of biodiversity (Suchanek 1994). Southern California and South Australia have high Elasmobranch diversity (Baker et al., 2008; Ebert 2003). California is home to over 65 species of Elasmobranch and South Australia has over 40 species inhabiting its coastline (Baker et al., 2008). Both locations are facing threats of anthropogenic stress to varying degrees. Southern California has a large human population leading to high concentrations of pollutants entering the marine environment (Bay et al., 2003; DiGiacomo et al. 2004). South Australia also faces threats of pollution entering the marine environment (Edwards et al., 2001;Leterme & Tuuri et al., 2023), but a significantly smaller human population eases this stress. South Australia, particularly the Gulf St. Vincent has unique oceanographic features like high salinity which differentiate it from the southern California coast (de Silva Samarasinghe et

al., 2003). Closely related Elasmobranchs in vastly different marine environments offer an opportunity to explore phylosymbiosis across global environments.

Here I use a reference independent approach to investigate microbiome similarity of 14 host species representing seven orders and two locations separated by 13,000 kilometers. I compare antimicrobial and heavy metal resistance genes on sharks and rays captured adjacent to large and small human population centers to investigate whether anthropogenic activity impacts heavy metal or antimicrobial genes or whether these genes are intrinsic to microbial life on the skin of Elasmobranchs. I constructed 90 Metagenome Assembled Genomes and tested the impact of host and environment on functional gene composition.

Methods

Sample Collection

We collected metagenomes from sharks or rays using "supersuckers" as described in Doane et al 2017. Briefly, four supersuckers, or blundt-end two-way syringes were filled with sterile seawater. When the plunger is depressed, sterile water rinses the microbes from the skin and is recollected. The microbial slurry is filtered through a 0.22 um Sterivex (Merek, Australia).

In California, sharks and rays were caught during research trawling operations with the California Department of Fish and Wildlife as described in Kerr et al., 2023. Trawls were deployed for 10 minutes at a time. Upon retrieval, catch was sorted, measured, and microbiomes were collected as described above. In South Australia, sharks and rays were caught during South Australian Research and Development Institute prawn trawling surveys (McLeay & Hooper 2024; Burnell et al., 2015) or using sein nets (for *M. tenuicaudatus* only) as described in Kerr et al., 2025 in review. Trawls were deployed for 30 minutes, upon retrieval, catch was sorted measured and microbiomes collected as described above.

Myliobatis tenuicaudatus were caught using sein nets by targeting one individual ray at a time at Seacliff Beach, South Australia. All microbiome samples were transported to the lab on ice and stored at –20 °C until DNA extraction.

All water samples were collected by filtering one liter of seawater through a 0.22 µm Sterivex. Water samples were transported on ice and stored at –20 °C until DNA extraction.

DNA Extraction and Sequencing

DNA was extracted from Sterivexes using the Macherey-Nagel NucleoSpin Tissue Kit following the protocol described in Kerr et al., 2023 for all samples. Extracted DNA was quantified with Qubit fluorometer (Invitrogen, USA). Four library preparation and sequencing methods were used. Subset A were library prepped using the Swift 2S Plus DNA Library Kit (now xGen DNA Library Kit by IDT). Samples were diluted to 1 ng/µL where possible and 50 µL of each sample was sonicated at 65 watts for 65 seconds at 20°C using the Covaris M220 Focused Ultrasonicator (Covaris, USA). Sonicated samples were library prepped with the Swift 2S Plus DNA Library Kit following manufacturer's instructions and sequenced on the Illumina MiSeq (350 bp) at San Diego State University (Illumina, USA). Sequences from this sequencing run are available on the SRA under BioProject PRJNA1299068. Subset B was sent as raw DNA to MIGs and sequenced on the Illumina NovaSeq (150 base pairs) (Illumina, USA) as described in Kerr et al., 2023. This data is available on the SRA under BioProject PRJNA837707. Subset C was sent to South Australian Genomics Center (SAGC) and library prepped using the Nextera kit (Illumina, USA) and sequenced on the MGI DNBSEQ-400 platform (150 bp) (MGI, Guangdong) available under BioProject PRJNA1303404. Subset D was library prepped as described in Kerr et al., In review using the QSonica and xGen DNA Library Kit by IDT kit (IDT, USA)

and sequenced by AGRF on the Illlumina NextSeq (Illumina, USA) and are available on the SRA under BioProject PRJNA1245075.

Bioinformatics

Metagenomes were quality controlled with fastp, removing adapter sequences, low quality reads, and reads with N bases using default parameters (Chen et al., 2018). MASH was used to assess sequence similarity using default parameters (Ondov et al., 2016). MASH distances of only the R1 sequences are used for comparison. MASH distances of 1 (completely different) and 0 (exactly the same) were removed from analysis to avoid comparisons of a sample to itself. Wilcox test was used to compare the MASH distances within and between host groups. MASH distances were plotted as an NMDS and between and within group distances were plotted as boxplots using R package ggplot2 (Wickham et al., 2016).

Megahit was used to co-assemble all metagenomes originating from a host species using paired end sequences and minimum contig length of 200 base pairs (Li et al., 2015). The argument `--presets meta-large` was used due to the high complexity of shark metagenomes (Doane et al., 2023; Kerr et al., 2023).

AMRFinderPlus was used to identify antimicrobial resistance and heavy metal resistance genes in each of the host and water column assemblies using default parameters (Feldgarden et al., 2021). The R package mvabund was used to compare the abundance of resistance genes across hosts and locations using a generalized linear model with negative binomial distribution. Based on the model, Anova was used to test for host or location specific differences (Wang et al., 2012).

Anvio metagenomics workflow was used for generating MAGs (Eren et al., 2021). Reads from all samples were mapped back to contigs from each co-assembly using Bowtie2 (Langmead and Salzberg 2012) and samtools (Danecek et al., 2012). Within Anvio, MetaBat2 was used for binning (Kang et al., 2019). CheckM was used to assess bin quality and only those with 75% completeness and less than 5% contamination were retained (Parks et al., 2014). All assembly and binning was conducted on the Flinders University HPC Deepthought (Flinders University). MAGs with greater than 70% completion and less than 10% contamination were uploaded to BVBRC for phylogenetic and gene function analysis (Olson et al., 2022). The Bacterial Genome Tree tool was used to create a phylogenetic tree of all MAGs with 50 genes, a maximum of 5 deletions and duplications (Olsen et al., 2022). Functional genes were analyzed at the level "subclass" for all analyses.

The abundance of functional genes was compared across bacterial taxonomy, host and location using a generalized linear model with negative binomial distribution in the mvabund package. Based on the model, Anova was used to test for bacterial phyla and the interaction of host or location (Wang et al., 2012).

Results

Sample Collection and Sequencing

A total of 156 metagenomes were collected from fourteen Elasmobranch species spanning seven orders. In addition, 17 water samples collected from California (n = 3) and South Australia (n = 14) were included in the analysis. The microbiomes from each host were assembled separately, and the sixteen co-assemblies resulted in 58,341,968 contigs with an average N50 of 584 base pairs (Table 6.1).

Table 6.1. Assembly results for each host species' co-assembly.

Host	Common Name	Location	Sample size	Contigs	N50 (bp)
Myliobatis californica	Bat ray	California	24	3,980,369	673
Urobatis halleri	Round ray	California	31	4,183,066	543
Gymnura marmorata	Butterfly ray	California	3	333,861	551
Squatina californica	Angelshark	California	1	208,630	451
Platyrhinoidis triseriata	Thornback guitarfish	California	3	326,216	587
Rhinobatos productus	Shovelnose guitarfish	California	1	248,628	413
Seawater		California	3	425,077	446
Myliobatis tenuicaudatus	Southern eagle ray	South Australia	11	5,487,185	1371
Heterodontus portusjacksoni	Port Jackson shark	South Australia	18	7,098,806	1109
Trygonorrhina dumerilii	Fiddler ray	South Australia	21	6,851,871	493
Dentiraja cerva	Whitespotted skate	South Australia	18	5,599,273	502
Hypnos monopterygius	Coffin ray	South Australia	1	497,226	420
Notorynchus cepedianus	Broadnose sevengill shark	South Australia	8	3,992,948	449
Aptychotrema vincentiana	Western shovelnose ray	South Australia	4	1,579,081	437
Squatina australis	Australian angelshark	South Australia	12	4,839,391	445
Seawater		South Australia	14	10,690,340	451

Metagenome (MASH) Similarity

The microbiomes from all Elasmobranch host orders except Torpediniformes showed significantly lower intraspecific order dissimilarity compared to interspecific order dissimilarity. Within an order microbiomes were more similar than those compared across orders (Heterodontiformes, Wilcox test $W = 49698$, $p < 0.001$; Hexanchiformes, $W = 48753$, $df = 1$, $p < 0.001$; Myliobatiformes, $W = 21672852$, $df = 1$, $p < 0.001$; Rajiformes, $W = 655444$, $df = 1$, $p < 0.001$; Rhinopristiformes, $W = 1906040$, $df = 1$, $p < 0.001$; Squaliformes, $W = 253467$, $df = 1$, $p < 0.001$; ; water, $W = 824700$, $df = 1$, $p < 0.001$; Torpediniformes $W = 4564$, $df = 1$, $p = 0.146$) (Figure 6.1). Elasmobranch microbiomes clustered separately from the water column microbiomes (Figure 6.2). Closely related hosts, especially the *Myliobatis* species (*M. californica* and *M. tenuicaudatus*) clustered together (Figure 6.2, Supplementary Figure 6.1).

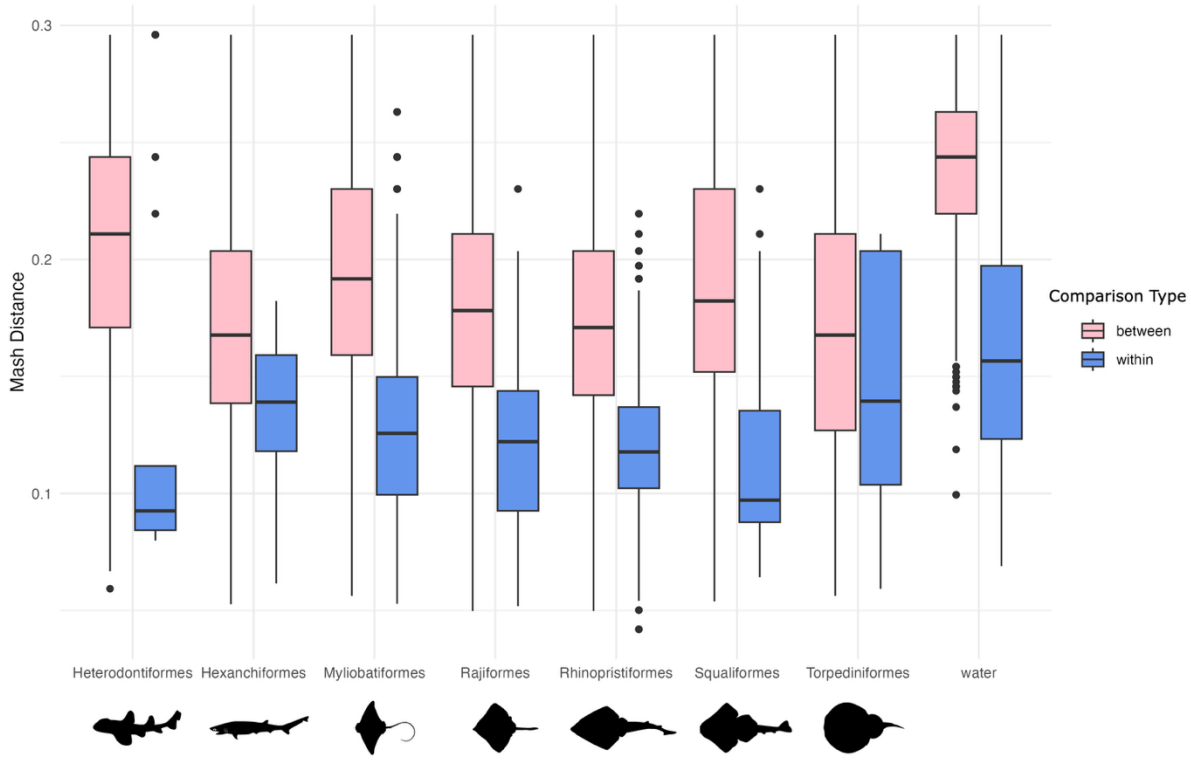

Figure 6.1: MASH distances for between and within species distances. Between group distances (pink) are significantly higher than within (blue) indicating microbiomes from the same host orders are more similar to each other than to microbiomes from different host orders.

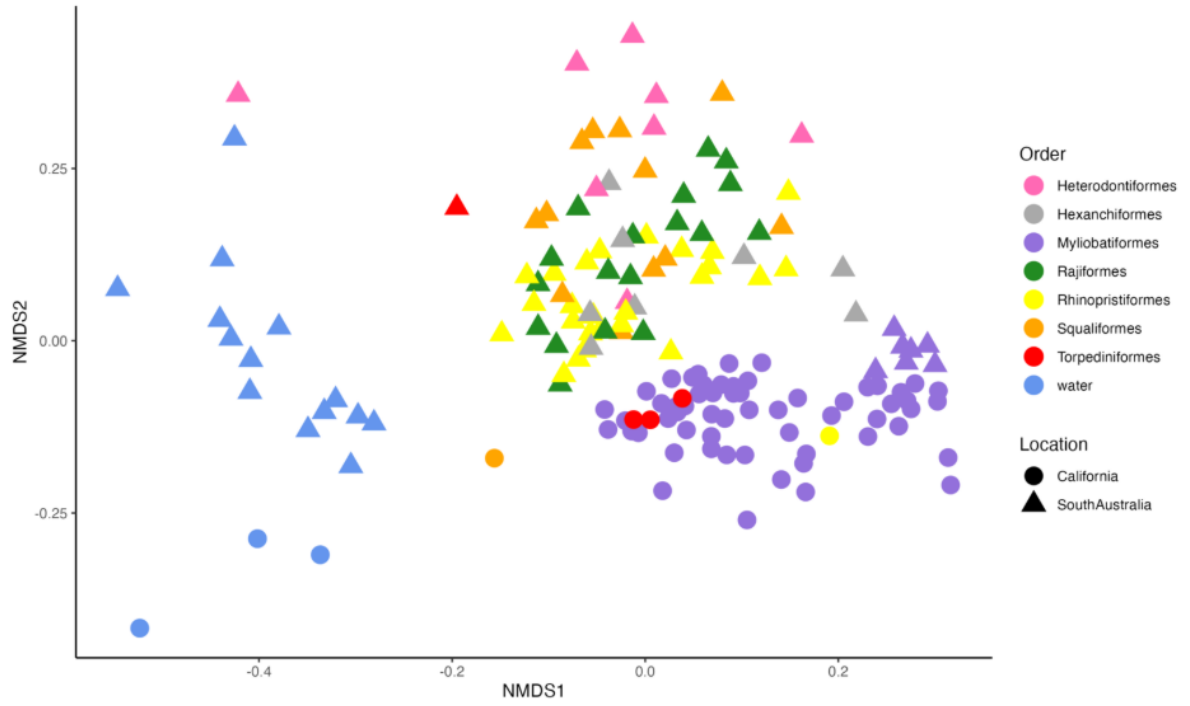

Figure 6.2: NMDS of MASH distances hosts are colored by host Order.

Antimicrobial and heavy metal gene distribution

AMR finder plus was used to identify antimicrobial and heavy metal resistance genes in each of the host assemblies. The MAGs constructed from the water column did not contain either antimicrobial or heavy metal resistance genes, regardless of whether the water was collected from South Australia and southern California. In contrast, microbes living on Elasmobranchs require a suite of resistance genes (Figure 6.3). Microbiomes on all hosts had genes associated with Mercury resistance, including high abundance of *merR, merT, merP, merC, merA , merG, merB, merB* and *merE*, suggesting that these genes have an essential role in the Elasmobranch microbiomes. Within Myliobatids, those sampled in California (*M. californica*) had a slightly higher abundance of resistance genes than *M. tenuicaudatus* sampled in South Australia. There was no significant effect of host or location on heavy

metal resistance gene abundance (Figure 6.3a). Host from both locations, for example *T. dumerilii*, *D. cerva*, and *U. halleri* had a variety of antibiotic resistance genes (Figure 6.3b). There was no significant effect of host or location on antimicrobial resistance gene abundance.

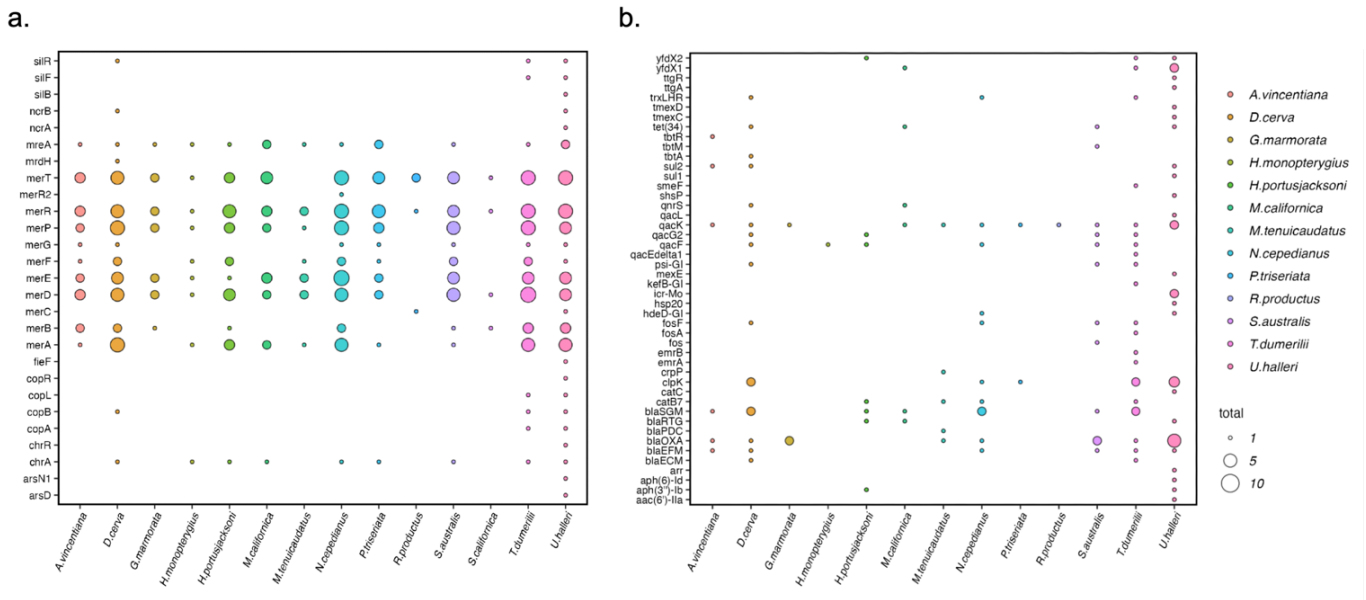

Figure 6.3: Abundance of a) heavy metal and b) antimicrobial resistance genes in host microbiomes. Bubbles represent the abundance of genes in each pathway identified by AMRfinderPlus in each host assembly.

Metagenome Assembled Genomes

A total of 90 MAGs were constructed, with the maximum number of MAGs (32) originated from *U. halleri*. MAGs belonged to eight phyla, over half (55) belonging to Pseudomonadota. Thirty-four genera are represented, and 19 MAGs could not be resolved to the genus level. Almost 80% of MAGs (70 out of 90 total) couldn't be identified to the species level. Archaeal MAGs (2) were only recovered from the south Australian water samples (Figure 6.4).

MAGs functional genes were significantly influenced by the interaction of host and bacterial phyla (ANOVA Dev = 1547, df = 13, p = 0.007) but not by the interaction of location and phyla (ANOVA Dev = 60, df = 1, p = 0.5).

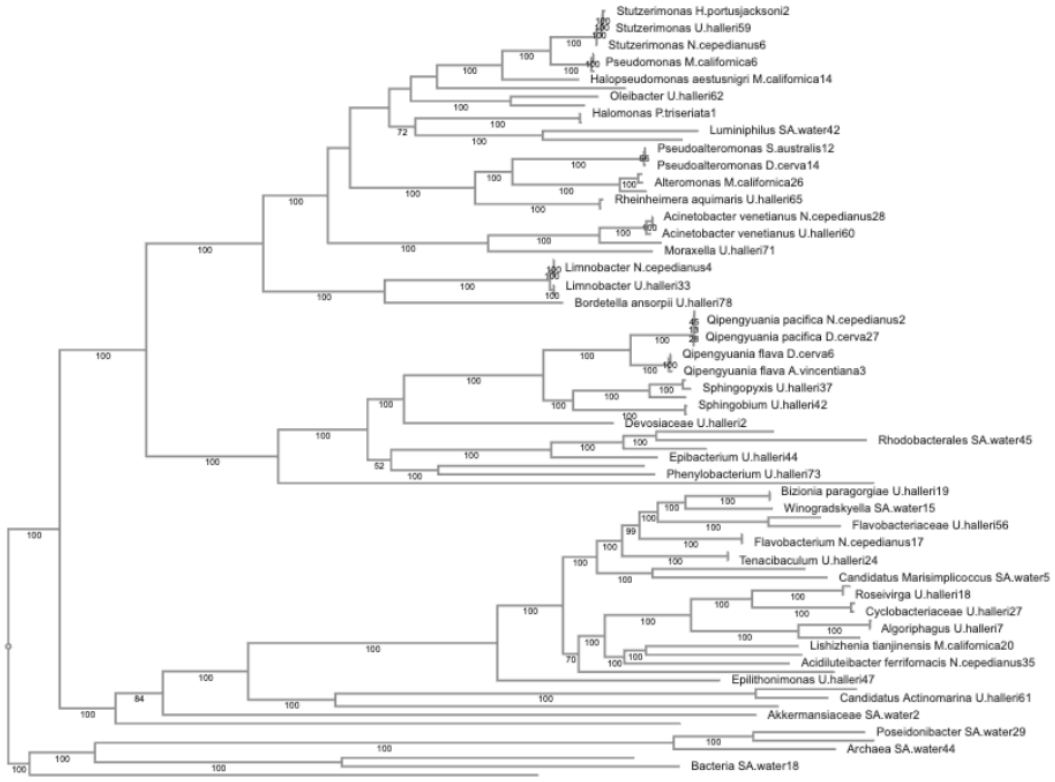

Figure 6.4: Phylogenetic tree of host-associated and water column MAGs. Nodes are labeled with bacterial taxon followed by host. Several MAGs were assembled from multiple hosts across both locations.

Table 2: Binning results for each host

Host	High Quality MAGs	Mean Completeness	Mean Contamination
Myliobatis californica	6	88.26	3.52
Urobatis halleri	32	90.18	3.39
Gymnura marmorata	2	97.89	1.41
Squatina californica	0		
Platyrhinoidis triseriata	5	77.46	4.79
Rhinobatos productus	0		
Seawater (California)	0		
Myliobatis tenuicaudatus	1	70.42	9.86
Heterodontus portusjacksoni	2	80.99	6.34
Trygonorrhina dumerilii	0		
Dentiraja cerva	9	87.32	3.60
Hypnos monopterygius	0		
Notorynchus cepedianus	8	86.27	1.94
Aptychotrema vincentiana	2	83.80	1.41
Squatina australis	4	89.79	3.52
Seawater (South Australia)	21	84.07	4.71

Discussion

Elasmobranch phylogeny is the primary driver of epidermal microbiomes in demersal hosts. Microbiomes of fourteen Elasmobranch hosts collected from southern California and southern Australia had high intraspecific similarity (Figure 6.1). Myliobatiformes demonstrate the strongest phylosymbiosis relationship, as two species living on different sides of the world have a more similar microbiome than distantly related species for other orders living in a shared environment (Figure 6.2). Heavy metal resistance genes are present in all host species and the mercury resistance (*mer*) pathway was consistently abundant across all hosts and both locations. Water column assemblies did not have any resistance genes detected at either location sampled (Figure 6.3). We constructed 90 MAGs, many of which were unable to be assigned to a species (Figure 6.4). Bacterial

phylum and host were the primary drivers of MAG functional gene abundance, but location was not significant. Overall, host phylogeny is the primary driver of metagenome similarity and MAG functional genes, indicating phylosymbiosis is maintained in Elasmobranchs even in species with prominent mucus.

Host was a significant driver of metagenome similarity within an order (Figure 6.1). Sharks typically have similar skin microbial communities within a species (Doane et al., 2020), while fish have more intraspecific variation (Chiarello et al., 2018; Pratte et al., 2018). Here we show that demersal Elasmobranchs, including rays and skates with high mucus coverage, have lower intraspecific variation, like pelagic sharks. Clustering of Myliobatiformes regardless of sampling location (Figure 6.2) is a strong indication of phylosymbiosis. Phylosymbiosis has been observed in Elasmobranchs microbiomes previously, but in fish phylosymbiosis is incomplete, potentially due to the mucus morphology of fish skin (Chiarello et al., 2018; Doane et al., 2020). This dynamic environment is hypothesized to increase microbiome variability similar to fish (Kerr et al., 2023). Rays and skates have a thicker mucus similar to teleost fish and reduced dermal denticles compared to sharks (Meyer & Seegers 2012) but still show a host specific relationship with their microbiomes (Clavere-Graciette et al., 2023; Kerr et al, 2023, in review). Here we show that despite mucus skin types having more dynamic microbial communities, host is still the primary driver of microbiome similarity (Figure 6.1, 6.2). Even when sampled from across the globe, Myliobatids cluster together, indicating host phylogeny is the primary driver of skin metagenome similarity in this clade (Figure 6.2). Denticle structure plays a significant role in structuring shark skin microbiomes (Goodman

et al., 2024), but other host factors such as mucus chemistry and the immune system are also driving a host specific microbiome.

Sequence similarity offers a reference independent approach to analyzing microbial communities (Ondov et al., 2016). Microbes associated with sharks are not well described in databases meaning a large proportion of sequences belong to unknown taxa (Kerr et al., 2023; Hesse et al., 2022). Our reference independent approach supports previous findings that within a host clade, microbiomes are more similar than across clades (Doane et al., 2020; Goodman et al., 2024; Kerr et al., 2023).

Resistance genes were present in all host microbiomes but were absent in the water column assemblies (Figure 6.3). Sharks typically have a higher abundance of heavy metal genes relative to the water column, hypothesized to support the offload of bioaccumulated heavy metals (Doane et al., 2017, 2021; Goodman et al., 2022; Kerr et al., 2023). Host and location did not play a significant role in resistance gene abundance. Hosts in this study are demersal and occupy mesotrophic niches, thus bioaccumulating heavy metals at similar rates (Matulik et al., 2017; Weijs et al., 2015). Similar exposure to heavy metals though diet may explain the similar abundance of resistance genes. Sharks feeding at a higher trophic level may cause a higher relative abundance of heavy metal resistance genes in their microbiomes (Doane et al., 2017). Interestingly, location did not play a role in the abundance of these genes, despite California and South Australia having vastly different human populations. Hosts with the fewest samples (n=1) had the lowest number of genes identified. High coverage is crucial for identifying AMR genes (Gweon et al., 2019). Due to the lower coverage of these assemblies compared to others with a greater number of samples, this is likely an underestimation of the true number of AMR and HMR genes in those assemblies.

Genes in the mercury resistance pathway (*mer*) were present in high abundance across many host species (Figure 6.3a). Mercury resistance appears to play an important role, and future culture dependent or transcriptomic studies could resolve the role these pathways play in Elasmobranch microbiomes.

Metagenome assemblies resulted in 90 Metagenome Assembled Genomes (MAGs) spanning 29 bacterial families. The abundance of functional genes in MAGs was significantly impacted by bacterial phyla. This is not unexpected as genetic traits are driven by bacterial phylogeny (Andersson et al., 2010, Greses et al., 2023) but can also be influenced significantly by ecology (Alneberg et al., 2020). The interaction of bacterial phyla and host significantly influenced functional gene abundance, while the interaction of bacterial phyla and location was not significant. In bacterioplankton MAGs functional gene composition was significantly driven by environmental gradients like salinity and depth (Alenberg et al., 2020). In Elasmobranch microbiomes host was a significant influence on functional gene diversity, indicating the skin microenvironment had more influence than the surrounding water column on functional gene abundance.

Almost 80% of MAGs could not be identified to the species level, similar to previous investigations of marine microbiomes (Khan et al., 2024) including Elasmobranchs (Doane et al., 2023; Kerr et al., 2023). The low annotation rates of single read sequencing data and metagenome assembled genomes highlight the unique uncharacterized marine bacteria. Several bacterial taxa were constructed across locations and host species (Figure 6.4). Some of these species, particularly *Erythrobacteraceae* (*Qipengyuania flava* and *Qipengyuania pacifica*) were assembled from five host species yet is not highly abundant in shark microbiomes (Doane et al., 2017, Goodman et al., 2023, Kerr et al., 2023; Pratte et al.,

2021). Metagenomic assemblies provide an opportunity to identify important microbes that may be overlooked due to low abundance in single read analysis of microbiome data. No MAG was assembled from all hosts, but more than half (47) of MAGs belonged to Gammaproteobacteria and Alphaproteobacteria (Figure 6.4). Gammaproteobacteria and Alphaproteobacteria are abundant members of Elasmobranch microbiomes, and likely occupy important niches (Black et al., 2021; Doane et al., 2023; Goodman et al., 2024; Kerr et al., 2023; Pogoreutz et al., 2024; Pratte et al., 2021).

One limitation of this design is that no host species was caught in two locations. Demersal species tend to be restricted to smaller ranges, *U. halleri* for example have distinct populations near Catalina Island and mainland California despite being only 35 km apart (Plank et al., 2010). Comparing the same species across large geographic distances or distinct populations would help decipher the contribution of host genetics on microbiome similarity within a species. Large migratory sharks like white sharks (*C. carcharias*) or whale sharks (*R. typus*) would be good candidates to further test environmental effects on host microbiomes in Elasmobranchs. Host phylogeny had a greater impact on gut microbiome similarity in *C. carcharias* and *R. typus* than diet (Pratte et al., 2022) and skin microbiomes likely reflect phylogenetic relationships.

Here we show that demersal Elasmobranch metagenomes and Metagenome Assembled Genome (MAG) functional genes are driven primarily by host phylogeny. Metagenome sequences were more similar within than between host orders (Figure 6.1). Myliobatiformes, especially the genus *Myliobatis* had high sequence similarity, even when sampled from California and South Australia (Figure 6.2). Resistance genes were present in all host metagenomes but were absent in the water column (Figure 6.3) indicating these are

genes required by Elasmobranch adapted microbes. Bacterial phylum and host were the primary drivers of MAG functional gene abundance, further supporting hosts are selecting a specific microbial community with unique functional gene potential., Our results indicate host phylogeny is the primary driver of microbiome similarity within a host clade, and that demersal Elasmobranchs maintain phylosymbiosis in their skin microbiomes, similar to their pelagic counterparts.

DISCUSSION

My thesis expands descriptions of Elasmobranch microbiomes by examining host-microbe relationships at different body sites, between Elasmobranch species, over time, and with individual bacteria. I collected over 150 microbiomes from sixteen species of wild Elasmobranchs and surrounding seawater. I used a combination of shotgun metagenomic sequencing to characterize their microbial communities and long read sequencing of bacterial isolates. I collected epidermal mucus to describe mucus carbohydrate composition using High Performance Liquid Chromatography (HPLC). I show that host and microbe form important relationships influenced primarily by host factors (body site, mucus composition, host phylogeny, age) but in specific circumstances also the environment (location, season).

I show that skin, gill, and cloaca provide unique microbial niches which are distinct between host species and that the bacterium *Photobacterium damselae* is found all Elasmobranch gut microbiomes and it contains novel prophages that are not present in any other environment. Juvenile Elasmobranchs demonstrate microbial flexibility through time, potentially enabling adaption to a dynamic shallow water in the gulf. I identified species-specific microbial communities in *M. californica* and *U. halleri* microbiomes even when hosts were sampled in a shared environment. I demonstrate ray microbial functions are similar to sharks, despite having reduced denticles and more prominent mucus. I describe the mucus chemistry of four species and identified microbial genes, which metabolize mucus compounds, identifying a host specific mechanism for microbial selection. I sequenced five bacterial isolates in pursuit of *P. damselae*, and although I did not isolate that strain, other Vibrio species in the gut contained prophages and plasmids similar to *P.*

damselae. Finally, I compared the epidermal microbiomes of fourteen species that live in the waters of South Australia and California and found that phylosymbiosis is the primary driver of microbiome similarity. Both single read and metagenome assembled genomes were driven by host, and location did not play a significant role in microbiome similarity or MAG functional gene abundance. To live on a shark or ray microbes need genes associated with carbohydrate utilization and heavy metal degradation, but not antimicrobial resistance genes.

Environmental influence on Elasmobranch microbiomes

In Chapter 1, I demonstrated that skin microbiomes of *M. tenuicaudatus* and *H. portusjacksoni* had similar functional potential despite differences in skin morphology. Therefore, microbial functions are required to live on elasmobranchs. While not measure the bacterioplankton in this chapter, Elasmobranch microbiome functions are consistently distinct from the water column microbiome (Doane et al., 2017, 2022; Goodman et al., 2022, 2024; Kerr et al., 2023). Elasmobranch skin microbiome functions are typically different across species (Goodman et al., 2024) but have not been previously compared in hosts sharing the same habitat.

Juvenile Elasmobranch microbiomes exhibit flexibility in taxonomic and functional composition (Chapter 2). While microbial communities were influenced by the environment, they remained taxonomically and functionally distinct from the water column bacterial community. This suggests that although the adaptive immune system is underdeveloped compared to adults, the innate immune system of Elasmobranchs is still selective of environmental bacteria. In Chapter 3, *Urobatis halleri* showed different taxonomic communities across two locations about 250 km apart. *Triakis semifasciata* also show taxonomic flexibility while maintaining functional potential over time (Doane et al., 2022).

Microbiome composition is also dynamic across habitats (Pogoreutz et al., 2019) and between wild and captive individuals (Clavere-Graciette et al., 2023; Gonçalves e Silva et al., 2020; Pinnell et al., 2021). Therefore, environmental factors like season or location influence taxonomic flexibility, but the host still exerts control over microbial functions in adults.

Host influences on Elasmobranch microbiomes

Host was the primary driver of Elasmobranch microbiomes. In Chapter 1 each host species has unique gill and cloaca microbial communities, indicating host specific structuring of internal microbiomes. Previous investigations showed taxonomic distinction between species and body sites (Clavere-Graciette et al., 2023; Pogoreutz et al., 2024; Storo et al., 2021). My data supports distinction of gut community between evolutionarily distinct species with similar diets, further supporting that gut microbiomes are driven by phylogeny rather than diet (Pratte et al., 2022). I also reconstructed a *Photobacterium damselae* bacterial genome which shared novel phage genes with other Elasmobranch *P. damselae* MAGs. These novel Elasmobranch specific prophage genes likely benefit the host by controlling populations of *P. damselae* (Knowles et al., 2016). The identification of a specific *P. damselae* strain in the gut of Elasmobranchs supports that this bacterium is an Elasmobranch specific microbe driven by host identity rather than diet, geographic location, or other environmental factors. In Chapter 5 I attempted to isolate *P. damselae*, but instead isolated three *Vibrio* and two *Shewanella* species. These strains also had numerous prophage genes, plasmids, and mobile genetic elements, which appears to be important to Elasmobranch-microbiome symbioses.

Elasmobranch dermal denticles have been well described with microscopy (Ankhelyi et al., 2018; Feld et al., 2019; Gabler-Smith et al., 2021), and replicated in 3D printed models (Chien et al., 2020). Denticle morphology influences epidermal microbial communities in sharks (Goodman et al., 2024). Less is known about the influence of mucus on Elasmobranch microbiomes, but mucus-microbe interactions have been previously described in fish (Dash et al., 2018; Reverter et al., 2018). Mucus composition of only three Elasmobranch species has been investigated (Bachar-Wikstrom et al., 2023a, 2023b). In Chapter 4, I describe the monosaccharide composition of four new species of Elasmobranch and identify microbial genes capable of recognizing and metabolizing host mucus carbohydrates.

Finally in Chapter 6, I describe phylosymbiosis in Elasmobranch epidermal microbiomes across the globe. Hosts from the same genus (and order) have more similar microbiomes even when sampled across the globe. Location did not play a significant role in shaping metagenome similarity, MAG functional gene abundance or resistance gene abundance. Host was the primary factor influencing MAG functional gene abundance and metagenomic similarity. Phylosymbiosis describes the pattern of closely related hosts having similar microbial communities compared to more distantly related hosts, but the underlying mechanisms driving this pattern are unknown in sharks. I suggest that mucus chemistry, denticle morphology, adaptive immune system and gut morphology are all important host factors that could explain phylosymbiosis in Elasmobranchs.

Limitations and Future Directions

A major challenge for non-model host microbiome studies is the sparsity of DNA databases. A large proportion of sequences (up to 30%) from the Elasmobranch microbiome

could only be identified to the Domain level - Bacteria, and an even larger proportion could not be matched to any sequence in the database, i.e., completely undescribed microbes. I addressed this by using database independent analyses, metagenomic assembly and sequencing isolates, but further characterization of novel marine bacteria is required to resolve of host-microbiomes. Future efforts to culture marine bacteria and long read sequencing of metagenomes would hugely improve the resolution of host microbiome studies and expand our understanding of host-microbiome interactions.

My thesis is observational in nature and cannot answer the same questions as experimental studies. I compare functional gene abundance across body sites, species and through time, but I did not address gene expression. Metagenomes and metatranscriptomes are correlated, although expression is more flexible than gene presence (Gilbert et al., 2010). Future studies could leverage metatranscriptomics to address the gap between gene presence and gene expression, especially under different laboratory conditions (eg, temperature, salinity, and pH). Alternatively, experiments utilizing cultured isolates growth on the monosaccharides I classified from the Elasmobranchs to identify bacteria that are specifically adapted to metabolizing host mucus compounds. Experiments on kelp-associated bacterial isolates (Busch et al., 2015) and kelp microbiomes (Qiu et al., 2019) have provided critical information on how microbiomes change under anthropogenic pressure. Future experiments would improve understanding of shark microbiomes under different conditions and would be useful in predicting the response to climate change and other anthropogenic pressures. The observational nature of my thesis did allow me to look at broad eco-evolutionary patterns in microbial communities in the wild to inform future experimental research.

Conclusion

My thesis adds to the growing body of literature describing host-microbe-environment interactions and identifies important underlying mechanisms for these relationships. I found that host is the primary driver of microbiomes in Elasmobranchs, while environment plays a minor role in shaping Elasmobranch microbiomes, primarily in juveniles. I identify phylosymbiosis in benthic rays and identify host factors (mucus chemistry, gut morphology, and immune system) that are contributing to the eco-evolutionary relationship of vertebrates that immerge early in the tree of life.

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APPENDICES

Appendix 1: Supplementary Material
Chapter 1

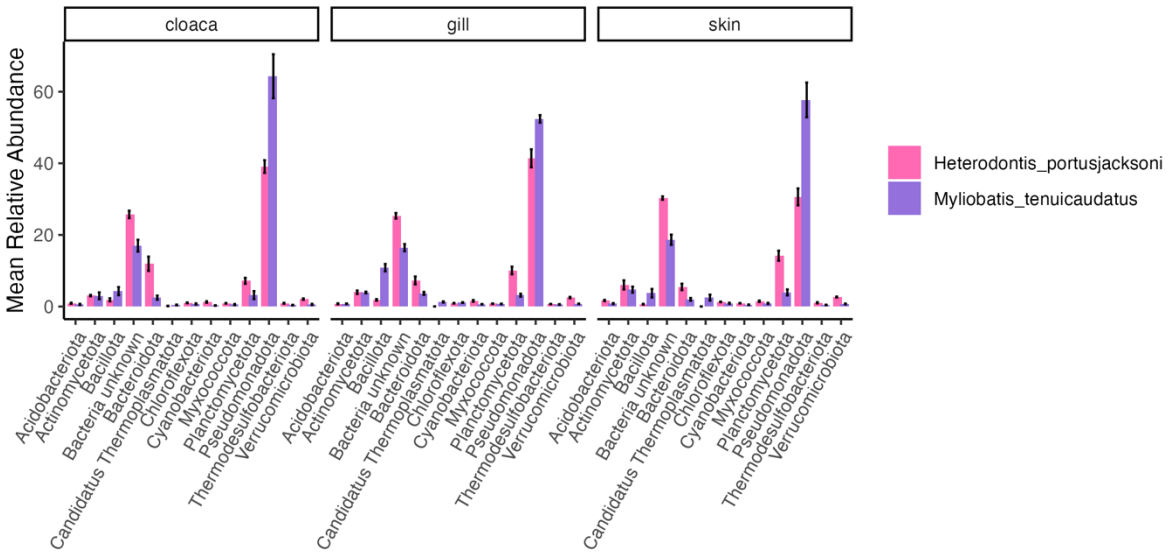
Supplementary Table 1.1: Sample sequencing statistics. Reads indicate the number of sequences from the metagenome which matched to the database.

SampleID	Reads	Species	Body site
EagleRay124c	58,457	*Myliobatis tenuicaudatus*	cloaca
EagleRay124d	40,377	*Myliobatis tenuicaudatus*	skin
EagleRay124g	46,143	*Myliobatis tenuicaudatus*	gill
EagleRay124w	544,358	*Myliobatis tenuicaudatus*	wound
EagleRay125c	79,372	*Myliobatis tenuicaudatus*	cloaca
EagleRay125d	338,342	*Myliobatis tenuicaudatus*	skin
EagleRay125g	36,881	*Myliobatis tenuicaudatus*	gill
EagleRay126d	55,391	*Myliobatis tenuicaudatus*	skin
EagleRay126g	9,727	*Myliobatis tenuicaudatus*	gill
EagleRay127c	12,660,969	*Myliobatis tenuicaudatus*	cloaca
EagleRay127d	585,825	*Myliobatis tenuicaudatus*	skin
EagleRay127g	114,530	*Myliobatis tenuicaudatus*	gill
EagleRay127v	1,047,623	*Myliobatis tenuicaudatus*	skin
EagleRay128c	7,817,235	*Myliobatis tenuicaudatus*	cloaca
EagleRay128d	934,839	*Myliobatis tenuicaudatus*	skin
EagleRay128g	33,172	*Myliobatis tenuicaudatus*	gill
EagleRay128v	60,086	*Myliobatis tenuicaudatus*	skin
EagleRay128w	25,204	*Myliobatis tenuicaudatus*	wound
EagleRay129c	198,597	*Myliobatis tenuicaudatus*	cloaca
EagleRay129d	37,382	*Myliobatis tenuicaudatus*	skin
EagleRay129g	19,084	*Myliobatis tenuicaudatus*	gill
EagleRay129v	65,643	*Myliobatis tenuicaudatus*	skin
EagleRay130c	23,054	*Myliobatis tenuicaudatus*	cloaca
EagleRay130d	316,842	*Myliobatis tenuicaudatus*	skin
EagleRay130g	10,600	*Myliobatis tenuicaudatus*	gill
EagleRay130v	40,609	*Myliobatis tenuicaudatus*	skin
EagleRay131c	110,751	*Myliobatis tenuicaudatus*	cloaca
EagleRay131d	12,748	*Myliobatis tenuicaudatus*	skin
EagleRay131g	62,368	*Myliobatis tenuicaudatus*	gill
EagleRay131v	599,078	*Myliobatis tenuicaudatus*	skin
EagleRay151c	129,459	*Myliobatis tenuicaudatus*	cloaca
EagleRay151d	32,950	*Myliobatis tenuicaudatus*	skin
EagleRay151g	36,115	*Myliobatis tenuicaudatus*	gill
EagleRay151v	118,969	*Myliobatis tenuicaudatus*	skin

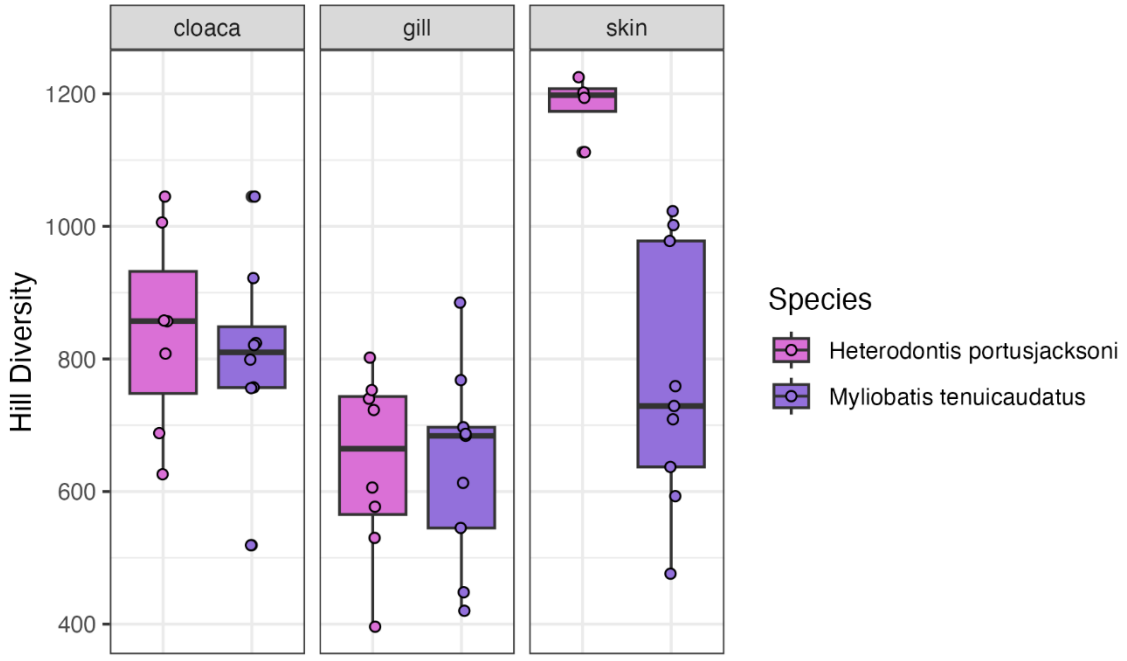
PortJackson132c	104,428	*Heterodontis portusjacksoni*	cloaca
PortJackson132g	10,854	*Heterodontis portusjacksoni*	gill
PortJackson132s	809,098	*Heterodontis portusjacksoni*	skin
PortJackson133g	65,481	*Heterodontis portusjacksoni*	gill
PortJackson135g	86,519	*Heterodontis portusjacksoni*	gill
PortJackson138c	89,280	*Heterodontis portusjacksoni*	cloaca
PortJackson140c	42,143	*Heterodontis portusjacksoni*	cloaca
PortJackson140g	26,026	*Heterodontis portusjacksoni*	gill
PortJackson140s	3,465,935	*Heterodontis portusjacksoni*	skin
PortJackson142c	125,454	*Heterodontis portusjacksoni*	cloaca
PortJackson142g	48,856	*Heterodontis portusjacksoni*	gill
PortJackson144c	416,599	*Heterodontis portusjacksoni*	cloaca
PortJackson144g	79,078	*Heterodontis portusjacksoni*	gill
PortJackson144s	3,510,634	*Heterodontis portusjacksoni*	skin
PortJackson146c	32,313	*Heterodontis portusjacksoni*	cloaca
PortJackson146g	99,862	*Heterodontis portusjacksoni*	gill
PortJackson148c	311,917	*Heterodontis portusjacksoni*	cloaca
PortJackson148g	44,628	*Heterodontis portusjacksoni*	gill
PortJackson148s	2,986,740	*Heterodontis portusjacksoni*	skin

Table: 1.2: Photobacterium damselae GenBank Accession numbers for database representatives.

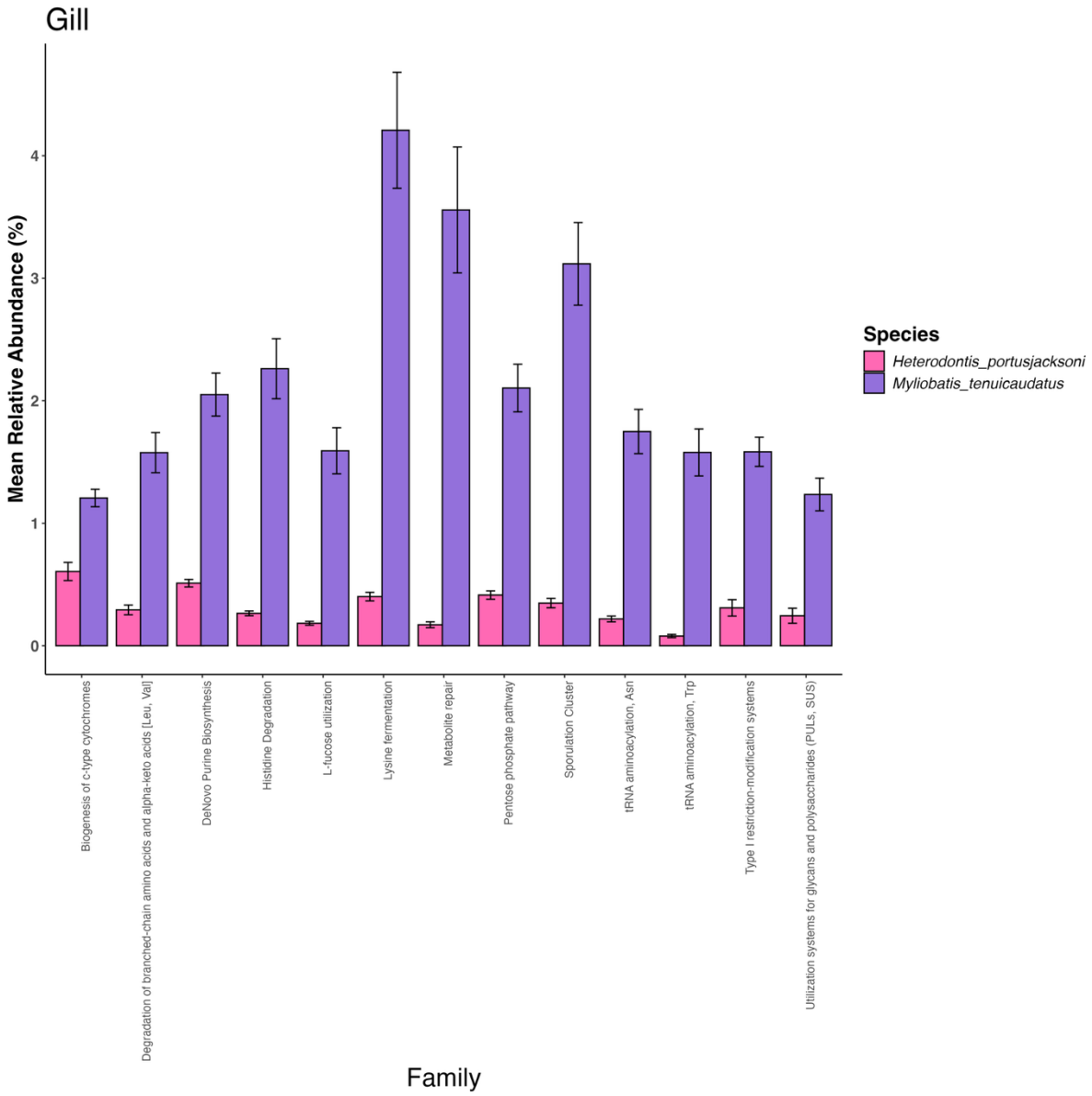
Strain	Isolation source	GenBank Accession
KC-Dl-1	Beluga whale (Delphinapterus leucas)	CP063049, CP063050, CP063048
AS-16-0963-3	Fish (Seriola lalandi)	CP065041, CP065042, CP065043
206352-6(DK32)	Rainbow trout (Oncorhynchus mykiss)	JABWTO000000000
A-162	Eel (Anguilla anguilla)	LZFN00000000
80077637	Human (Homo sapiens)	WAEO00000000



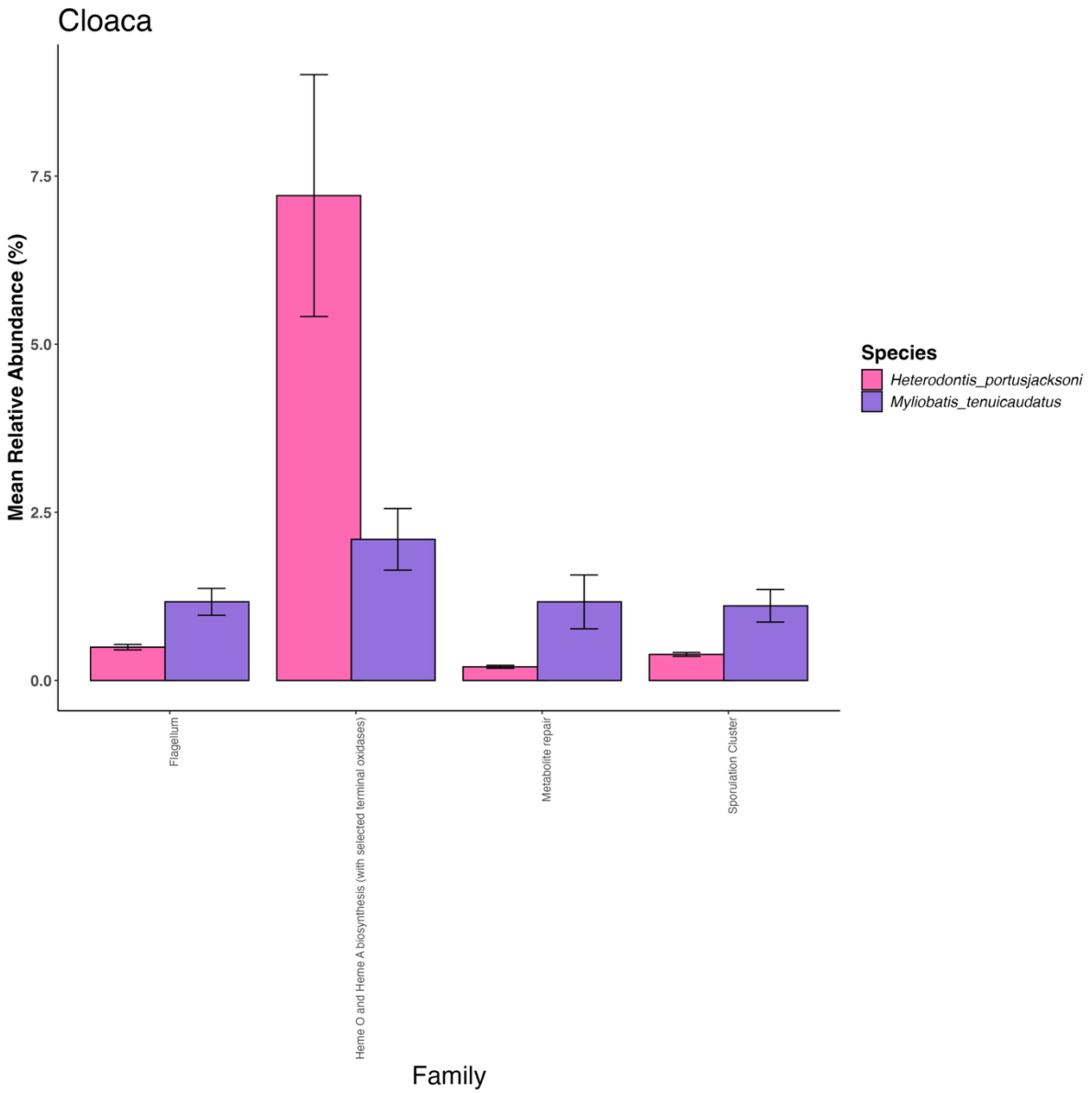
Supplementary Figure 1.1: Mean relative abundance of the ten most abundant phyla in *M. tenuicaudatus* and *H. portusjacksoni* microbiomes. Mean was calculated for each body site within a host. Where one bar is present that taxa was not in the 10 most abundant phyla at that body site. Error bars represent standard error.



Supplementary Figure 1.2: Hill Diversity for each body site. Dots represent individual samples; boxes represent the inner quartile range.

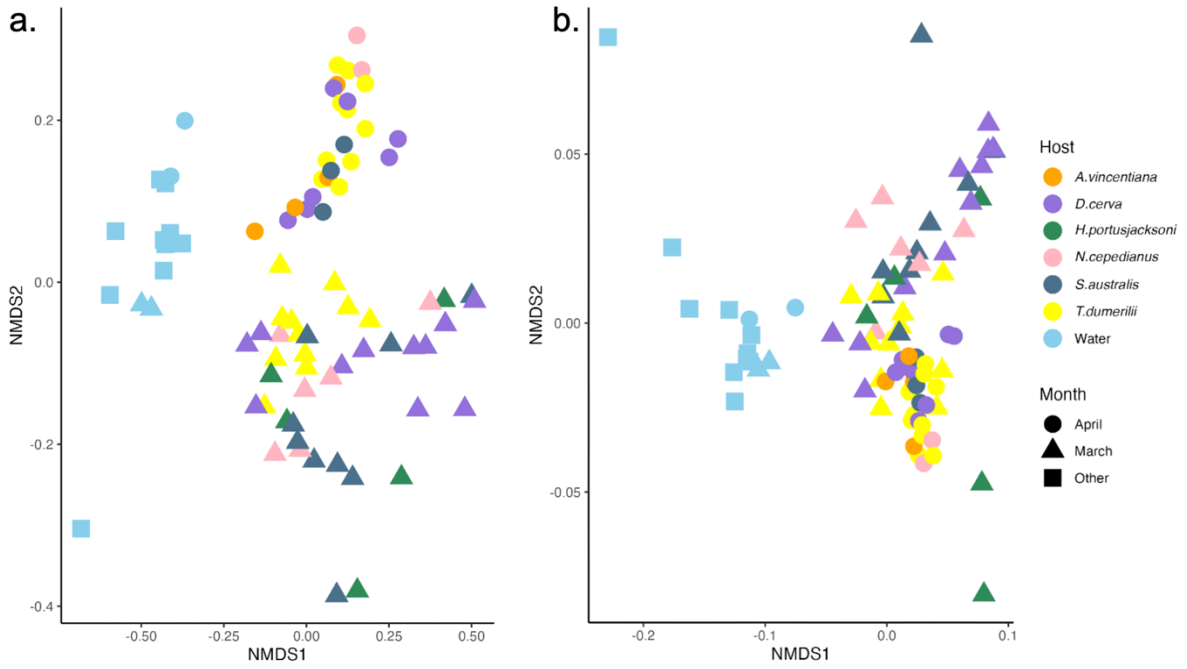


Supplementary Figure 1.3: Abundant (greater than 1% abundance) and significantly different functional genes in *M. tenuicaudatus* and *H. portusjacksoni* gill microbiomes.



Supplementary Figure 1.4: Abundant (greater than 1% abundance) and significantly different functional genes in *M. tenuicaudatus* and *H. portusjacksoni* cloaca microbiomes.

Chapter 2



Supplementary Figure 2.1: NMDS of a.) family and b.) functional gene microbial communities for host and water column.

Chapter 3

Supplementary Table 3.1: Sequencing characteristics for all metagenomes.

Sample	Number of Sequences	Total Length (base pairs)	SRA Accession Number
BR1_*M. californica*_LA	2,836,348	417,583,808	SRR19392812
BR2_*M. californica*_LA	3,087,737	412,648,462	SRR19392811
BR3_*M. californica*_LA	3,222,884	429,435,652	SRR19392800
BR4_*M. californica*_LA	2,366,977	348,943,221	SRR19392789
FLABR32_*M. californica*_LA	3,270,999	481,363,353	SRR19392784
FLABR36_*M. californica*_LA	4,455,194	585,640,485	SRR19392783
BR6_*M. californica*_SD	3,874,957	527,003,205	SRR19392782
FCBR13_*M. californica*_SD	3,039,185	404,934,699	SRR19392781
FCBR14_*M. californica*_SD	4,302,024	543,925,651	SRR19392780
FCBR15_*M. californica*_SD	2,641,366	389,418,541	SRR19392779
FCBR16_*M. californica*_SD	4,970,087	626,033,720	SRR19392810
FCBR18_*M. californica*_SD	3,767,721	486,296,771	SRR19392809
FDPBR1_*M. californica*_SD	2,645,824	350,666,845	SRR19392808
FSOBR2_*M. californica*_SD	3,923,330	505,311,011	SRR19392807
FSOBR3_*M. californica*_SD	3,158,635	421,845,873	SRR19392806

FLARR20_*U. halleri*_LA	3,892,074	503,682,608	SRR19392805
FLARR21_*U. halleri*_LA	3,262,778	419,680,365	SRR19392804
FLARR22_*U. halleri*_LA	3,184,239	418,069,390	SRR19392803
FLARR25_*U. halleri*_LA	4,672,533	580,369,873	SRR19392802
FLARR30_*U. halleri*_LA	4,133,388	513,704,828	SRR19392801
FLARR42_*U. halleri*_LA	3,957,020	542,437,760	SRR19392799
FLARR44_*U. halleri*_LA	3,673,869	503,817,610	SRR19392798
RR1_*U. halleri*_LA	5,499,533	749,123,698	SRR19392797
RR3_*U. halleri*_SD	3,598,286	449,710,240	SRR19392796
RR4_*U. halleri*_SD	5,204,519	707,235,278	SRR19392795
RR5_*U. halleri*_SD	4,233,567	592,387,351	SRR19392794
RR7_*U. halleri*_SD	4,188,561	581,233,486	SRR19392793
RR8_*U. halleri*_SD	4,533,582	605,911,450	SRR19392792
FCRR11_*U. halleri*_SD	2,667,563	352,045,492	SRR19392791
FCRR12_*U. halleri*_SD	3,625,315	470,974,465	SRR19392790
FCRR6_*U. halleri*_SD	4,456,501	614,810,049	SRR19392788
LAW43 water	481,196	131,375,559	SRR19392785
SDBW1 water	76,839	20,277,138	SRR19392787
SDBSW2 water	493,930	139,108,939	SRR19392786

Supplementary Table 3.2: ANOVA and Tukey-Kramer results for Level 3 SEED Subsystem comparisons between host microbiomes and the water column.

Gene	ANOVA p-value	Multiple R-squared	*M. californica* Tukey-Kramer p-value	*U. halleri* Tukey-Kramer p-value
Ammonia assimilation	< 0.001	0.71	< 0.001	< 0.001
Bacterial Chemotaxis	< 0.001	0.51	0.003	0.001
Bacterial hemoglobins	0	0.32	< 0.001	< 0.001
Branched-Chain Amino Acid Biosynthesis	< 0.001	0.78	< 0.001	< 0.001
Choline and Betaine Uptake and Betaine Biosynthesis	< 0.001	0.77	< 0.001	< 0.001
Cobalt-zinc-cadmium resistance	< 0.001	0.75	< 0.001	< 0.001
Copper homeostasis	< 0.001	0.66	< 0.001	< 0.001
De Novo Purine Biosynthesis	< 0.001	0.79	< 0.001	< 0.001
DNA-replication	< 0.001	0.42	0.04	< 0.001
Fatty Acid Biosynthesis FASII	< 0.001	0.43	< 0.001	0.001
Flagellum	< 0.001	0.45	< 0.001	< 0.001
Multidrug Resistance Efflux Pumps	< 0.001	0.53	< 0.001	< 0.001
Phosphate metabolism	< 0.001	0.37	0.006	< 0.001
Potassium homeostasis	< 0.001	0.54	< 0.001	< 0.001
Respiratory Complex I	< 0.001	0.29	0.3	0.01

RNA Polymerase III	< 0.001	0.37	0.04	0.98
Terminal cytochrome C oxidases	0	0.33	0.06	0.96
Ton and Tol transport system	< 0.001	0.39	0.01	< 0.001

Table 3.3: Average nucleotide identity between each stingray MAG and most similar genome identified by PATRIC's Similar Genome Finder.

Reference Genome	Query	GenBank Accession Number	Percent Identity
Caulobacteraceae	Bin_9	DEWY01000001.1	80.7945
Alcanivorax	Bin_16	DFMU01000001.1	97.2609
Rheinheimera aquimaris	Bin_17	PNRD01000001.1	98.384
Rhodobacteraceae	Bin_31	JAMWZG010000001.1	77.9628
Enhydrobacter	Bin_33	LR733345.1	96.2021

Chapter 4

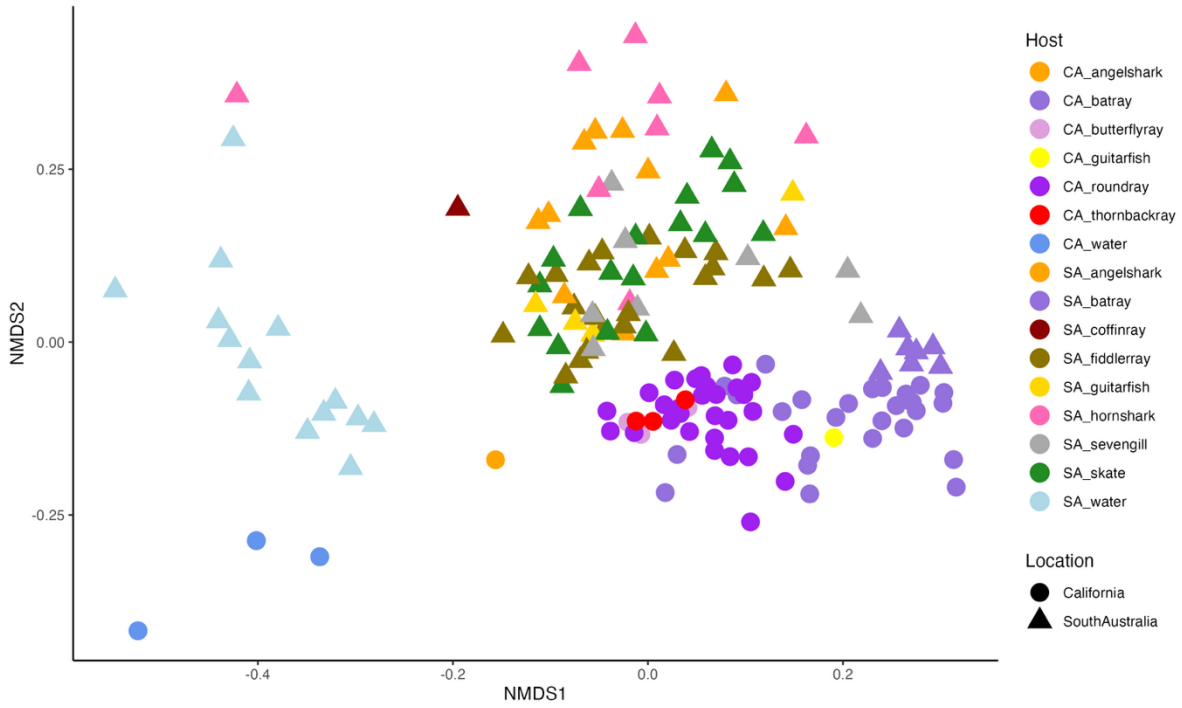
Supplementary Table 4.1: Host metadata. N indicates samples where mucus from male and female individuals was combined. Pooled samples include mucus from 2-4 individuals.

Sample	Host	Sex	Type
ER129	EagleRay	Female	Individual
ER131	EagleRay	Female	Individual
ER151	EagleRay	Female	Individual
ER247	EagleRay	Male	Individual
ERGSV303	EagleRay	Female	Individual
ER128130	EagleRay	Female	Pool
GSV322B	Skate	Male	Individual
GSV325	Skate	Female	Individual
GSV336	Skate	Female	Individual
GSV337	Skate	Female	Individual
GSV339	Angelshark	Male	Individual
GSV401	PortJacksonShark	Male	Individual
GSV416	FiddlerRay	Female	Individual
SkatePool1	Skate	N	Pool
SkatePool2	Skate	N	Pool
SkatePool3	Skate	N	Pool
AngelsharkPool1	Angelshark	Female	Pool
AngelsharkPool2	Angelshark	Female	Pool

Supplementary Table 4.2 Dunn's test results for pairwise comparisons of monosaccharide abundance.

Monosaccharide	Comparison	Z	p-value	p-adjusted
Arabinose	Angelshark-EagleRay	0.0000	1.000	1.000
	Angelshark-Skate	-1.695	0.090	0.135
	EagleRay-Skate	-2.076	0.037	0.11
Fucose	Angelshark-EagleRay	-0.581	0.561	0.841
	Angelshark-Skate	0.053	0.958	0.957
	EagleRay-Skate	0.777	0.437	1.000
Galactose	Angelshark-EagleRay	-2.055	0.039	0.119
	Angelshark-Skate	-1.107	0.268	0.268
	EagleRay-Skate	1.162	0.245	0.368
Glucose	Angelshark-EagleRay	-2.583	0.009	0.294
	Angelshark-Skate	-1.107	0.268	0.268
	EagleRay-Skate	1.807	0.071	0.106
Glucuronic Acid	Angelshark-EagleRay	-1.695	0.090	0.135
	Angelshark-Skate	0.000	1.000	1.000
	EagleRay-Skate	1.076	0.038	0.113
Glucosamine	Angelshark-EagleRay	-2.127	0.033	0.050
	Angelshark-Skate	0.132	0.894	0.894
	EagleRay-Skate	2.768	0.006	0.017
Mannose	Angelshark-EagleRay	-2.378	0.017	0.026
	Angelshark-Skate	-0.297	0.766	0.766
	EagleRay-Skate	2.549	0.011	0.324
Ribose	Angelshark-EagleRay	-2.353	0.0186	0.027
	Angelshark-Skate	0.000	1.000	1.000
	EagleRay-Skate	2.882	0.004	0.012

Chapter 6



Supplementary 6.1: NMDS of MASH distances for host and water column metagenomes. Hosts are colored by genus, Myliobatis genus cluster close together despite being sampled from across the globe.

APPENDIX 2: ADDITIONAL MANUSCRIPTS

Elasmobranchs Exhibit Species-Specific Epidermal Microbiomes Guided by Denticle Topography

Abstract

Elasmobranch epidermal microbiomes are species-specific, yet microbial assembly and retainment drivers are mainly unknown. The contribution of host-derived factors in recruiting an associated microbiome is essential for understanding host-microbe interactions. Here, we focus on the physical aspect of the host skin in structuring microbial communities. Each species of Elasmobranch exhibits unique denticle morphology, and we investigate whether microbial communities and functional pathways are correlated with the morphological features or follow the phylogeny of the three species. We extracted and sequenced the DNA from the epidermal microbial communities of three captive shark species: Horn (*Heterodontus francisci*), Leopard (*Triakis semifasciata*), and Swell shark (*Cephaloscyllium ventriosum*) and use electron microscopy to measure the dermal denticle features of each species. Our results outline species-specific microbial communities, as microbiome compositions vary at the phyla level; *C. ventriosum* hosted a higher relative abundance of Pseudomonadota and Bacillota, while *H. francisci* were associated with a higher prevalence of Euryarchaeota and Aquificae, and Bacteroidota and Crenarchaeota were ubiquitous with *T. semifasciata*. Functional pathways performed by each species' respective microbiome were species-specific metabolic. Microbial genes associated with aminosugars and electron-accepting reactions were correlated with the distance between dermal denticles, whereas desiccation stress genes were only present when the dermal denticle overlapped. Microbial genes associated with Pyrimidines, chemotaxis and virulence

followed the phylogeny of the sharks. Several microbial genera display associations that resemble host evolutionary lineage, while others had linear relationships with interdenticle distance. Therefore, denticle morphology was a selective influence for some microbes and functions in the microbiome contributing to the phylosymbiosis.

Importance

Microbial communities form species-specific relationships with vertebrate hosts, but the drivers of these relationships remain an outstanding question. We explore the relationship between a physical feature of the host and the microbial community. A distinguishing feature of the subclass Elasmobranchii (sharks, rays, and skates), is the presence of dermal denticles on the skin. These structures protrude through the epidermis providing increased swimming efficiency for the host and an artificial model skin affect microbial recruitment and establishment of cultured microbes but has not been tested on natural microbiomes. Here, we show some naturally occurring microbial genera and functional attributes were correlated with dermal denticle features, suggesting they are one, but not only contributing factor in microbiome structure on benthic sharks.

Introduction

The coupling of the eukaryotic host and its respective microbial communities ("microbiome") has been reclassified as a meta-organism, representing the mutualistic dependence between a multicellular organism and its respective microbial communities[1]. Factors such as host age[2], life history [3], diet [4,5], environment [6,7], and body site [8] contribute to the composition of microbial communities to varying extents, both internally (e.g., oral, gut, reproductive tracts, etc.) and externally (integumentary system, i.e. skin). As the largest organ of any body, the skin of a host is implicated in numerous facets of host health with the

outermost layer of skin, the epidermis, providing a non-invasive avenue to investigate skin health and disease of a host. However, most epidermal microbiome studies focus largely on mammals [9,10], particularly humans [11]. Therefore, characterizing the external microbiomes of non-humans is increasingly necessary to understand how the recruitment and retainment of microbial communities leads to disease.

The epidermal microbiome of different marine vertebrates such as teleost fish, cartilaginous fish (Elasmobranchs), and marine mammals contains core microbial species that are conserved throughout their geographic regions and genetically distinction from the surrounding environment[4,12]. Whale sharks (*Rhincodon typus)* from sub-tropical locations around the world share a core microbiome, including the genera *Rheinheimera, Leeuwenhoekiella, Algoriphagus, Sphingoblium, Aeqorivita,* and *Flavobacterium*[5]. Humpback whales (*Megaptera novaeangliae*) found across the northern Pacific, have a distinct set of microbial species including *Psychrobacter*, *Tenacibaculum*, uncultured *Moraxellaceae*, *Flavobacterium*, *Flavobacteriaceae*, and *Gracilibacteri*[13]. Despite the highly diverse surface features of these ocean organisms, each possess unique mechanisms to recruit and maintain respective core microbes. Fish dermis, for instance, feature goblet cells that produce a thick layer of nutrient-rich mucus to cover dermal surfaces[14], while marine mammals often shed their skin to deter biofilm formation[13]. Elasmobranchs have dermal denticles that protrude through the epidermis to aid in hydrodynamics, while possessing a reduced amount of mucus. These host characteristics exhibit a selective effect with respect to epidermal microbiome structure: the physical characteristic of the shark denticle topography aids in reducing fluid friction and deter biofilm formation while physiological characteristics of the mucus layer provides antimicrobial properties.

The epidermal microbiomes of sharks, which have densely packed denticles, are highly shared across individuals of the same species, while the epidermal microbiomes of stingray, which have sparse dermal denticles and thick mucus, are more variable, suggesting the interaction of dermal denticles and microbiome characteristics [15,16]. Thus, while microbes pervasively associated with the epidermis of fishes throughout the host's evolutionary history, describing the specific microbial species present on the skin of marine vertebrates and the host factors influencing microbiome recruitment and retainment is required.

The aforementioned host-microbiota relationships are a product of phylosymbiosis, an outlined trend whereby associated microbial communities are deterministically assembled by host phylogeny across evolutionary time[17]. Examples of these evolutionarily persistent associations include those between coral reef invertebrates[18], fish[4,19], and humans [20], and their respective microbiomes. A long-standing relationship between a host and microbiome is one that exist between that of sharks and their epidermal microbiome; having an extensive evolutionary history has allowed sharks to adapt alongside a recruited and maintained microbiome and these phylosymbiotic trends occur across shark species including leopard (*Triakis semifasciata*), thresher (*Alopias vulpinus*), blacktip reef (*Carcharhinus melanopterus*), nurse (*Ginglymostoma cirratum*), tiger (*Galeocerdo cuvier*), lemon (*Negaprion brevirostris*), sandbar (*Carcharhinus plumbeus*), Caribbean reef (*Carcharhinus perezii*), and whale sharks (*Rhincodon typus*)[8,19,21–23]. The host-related factors that impact the phylosymbiotic relationship between epidermal microbiomes and shark hosts, remains an outstanding question.

We previously reported the principle that captive, aquatic Elasmobranch species maintain a comparable epidermal microbiome to wild counterparts[16]. The nearby proximity of captive Elasmobranchs sampled in this study provided an opportunity to characterize the epidermal microbiomes associated with *T. semifasciata*, *H. francisci*, and C. *ventriosum* populations and test whether microbial patterns in benthic shark epidermal microbiomes mirror host phylogeny and correlate with denticle morphology. These sharks were chosen as our model because the hosts are phylogenetically distinct, having an evolutionary distance of approximately 200 million years between *H. francisci* (Heterodontiformes) and both *T. semifasciata* and *C. ventriosu*m (Carcharhiniform)[24] (Figure 1), are relatively small, can be easily obtained from captive sources, and possess unique dermal denticle topography. We aim to explore whether denticle topography is a feature of the host that is influencing the microbial community and a mechanism underlying emergent patterns of phylosymbiosis. We hypothesize the taxonomic composition of epidermal microbiomes belonging to the benthic Elasmobranch species will show correlations both with overall host phylogeny and specific individual microbes. We anticipate the evolutionary lineage of each host species, reflected through alterations in the composition of the microbiome, will also be modulated by the topographical characteristics of the dermal denticles, albeit with weaker covariance.

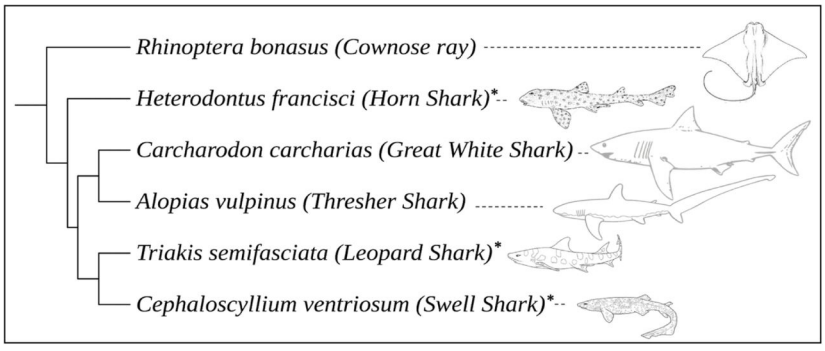


Figure 1: Phylogeny of a subset of Elasmobranch species to highlight the relationships of the species investigated in this paper (denoted with an asterisk).

Results

The epidermal microbiomes belonging to three benthic shark species, *T. semifasciata* (n=11), *H. francisci* (n=10), and *C. ventriosum* (n=10) were sampled in captivity (Table 1) alongside water column samples. Water-associated microbiomes collected from the captive environment were statistically dissimilar to host microbiomes in PERMANOVA main group tests (PERMANOVA: Genus, Pseudo-$F_{df = 1, 33} = 3.85$, P(perm) < 0.05) and pairwise analyses ($p < 0.05$), did not influence host-associated microbiomes, and are not investigated further.

Table 1: Metadata for sampled Elasmobranch epidermal microbiomes. Shark species abbreviated as follows: Heterodontus francisci (HS, Horn Shark), *Triakis semifasciata* (LS, Leopard Shark), and Cephaloscyllium ventriosum (SS, Swell Shark). Sampling environments designated in sample name as Birch Aquarium (BA) or National Oceanic and Atmospheric Administration (NOAA).

Host Species	Sample Name	Year	Sex	BP	Sequences
Heterodontus francisci	HS BA 1	2018	Female	105,565,799	5,217,911
	HS BA 2	2018	Male	82,214,142	2,876,236
	HS BA 3	2018	Female	74,359,847	454,887
	HS NOAA 1	2020	Female	79,764,450	540,818
	HS NOAA 2	2020	Female	34,456,117	108,399
	HS NOAA 3	2020	Female	165,544,580	995,733
	HS NOAA 4	2020	Female	143,548,493	508,012
	HS NOAA 5	2020	Female	133,890,137	260,273
	HS NOAA 6	2020	Female	112,568,752	2,626,021
	HS NOAA 7	2020	Female	165,377,448	512,408
	HS NOAA 8	2020	Female	296,267,509	441,426
Triakis semifasciata	LS BA 1	2018	Female	13,254,680	1,470,279
	LS BA 2	2018	Female	165,572,548	295,852
	LS BA 3	2018	Male	129,410,990	516,511
	LS BA 4	2018	Female	140,813,416	261,381
	LS BA 5	2019	Female	814,847,485	3,179,134
	LS BA 6	2019	Female	494,586,101	246,558
	LS BA 7	2019	Female	559,061,883	410,944
	LS BA 8	2019	Female	451,224,580	2,592,630
	LS BA 9	2019	Female	877,376,851	563,299
	LS BA 10	2019	Female	438,923,920	429,758
Cephaloscyllium ventriosum	SS BA 1	2018	Female	110,491,568	4,549,152
	SS BA 2	2018	Female	104,513,010	36,040
	SS BA 3	2018	Female	146,650,534	360,783
	SS BA 4	2018	Female	162,351,778	490,856
	SS BA 5	2018	Female	147,803,072	447,888
	SS BA 6	2018	Male	170,635,774	517,078
	SS BA 7	2018	Male	170,164,874	320,471
	SS BA 8	2018	Male	175,053,487	624,057
	SS BA 9	2018	Male	502,674,714	402,315
	SS BA 10	2018	Male	1,097,190,642	445,369

The total number of bacterial families present for each shark's microbiome ranged from 164 to 205 individuals associated with *T. semifasciata*, 176 to 205 with *H. francisci*, and 200 to 206 with *C. ventriosum*. To evaluate the overall diversity each of each shark species, the alpha-diversity of the epidermal microbiomes were compared and no differences of microbial community richness (Margalef's *d*), evenness (Pielou's *J'*), or overall diversity (Inverse Simpson (1-l)) were observed across shark species (Welch's t-test; $p > 0.05$; Table

2) at each taxonomic level (class, family, and genus). The greatest differences observed were measures of richness at genus level, as *C. ventriosum* were recorded to harbor the highest average overall diversity (8.88 ± 1.8 S.D.) followed by *T. semifasciata* (6.36 ± 1.43), and finally *H. francisci* (5.51 ± 1.91).

Table 2: Average alpha-diversity metrics of richness, evenness, and overall diversity for epiedermal microbiome communities associated with *T. semifasciata,H. francisci,* and *C. ventriosu*m

Host Species	Margalef's (*d*) Index ± S.D.	Pielou's (*J'*) Index ± S.D.	Inverse Simpson (1-λ) Index ± S.D.
T. semifasciata	6.36 ± 1.43	0.999 ± 1.4E-04	0.985 ± 0.002
H. francisci	5.51 ± 1.91	0.999 ± 9.5E-05	0.979 ± 0.003
C. ventriosum	8.88 ± 1.8	0.999 ± 5.9E-05	0.992 ± 0.001

The benthic sharks microbiome hosted a diversity of bacterial phyla, with the following showing an average relative abundance greater than 1.0%: Euryarchaeota (5.0 ± 1.47 % S.D.; Figure 2), Tenericutes (2.76 ± 0.75 %), Pseudomonadota (2.41 ± 8.82 %), Bacillota (2.37 ± 1.76 %), Actinobactria (2.17 ± 1.26 %), Bacteroidota (1.59 ± 1.97 %), Cyanobacteria (1.58 ± 0.59 %), Aquificae (1.32 ± 0.55 %), Crenarchaeota (1.3 ± 0.9 %), and Spirochaetes (1.23 ± 1.3 %). While each host species harbored the same phyla, the relative abundance varied with *C. ventriosum* retaining the highest relative abundance of Pseudomonadota (39.1 ± 3.25 %) and Bacillota (9.1 ± 3.6 %), while *H. francisci* harbored highest relative abundances of Euyarchaeota (24.5 ± 10.8 %), and Tenericutes (10.8 ± 2.2 %), and *T. semifasciata* highest relative abundance of Actinomycetota (6.32 ± 1.45 %) and Crenarchaeota (5.61 ± 2.67 %).

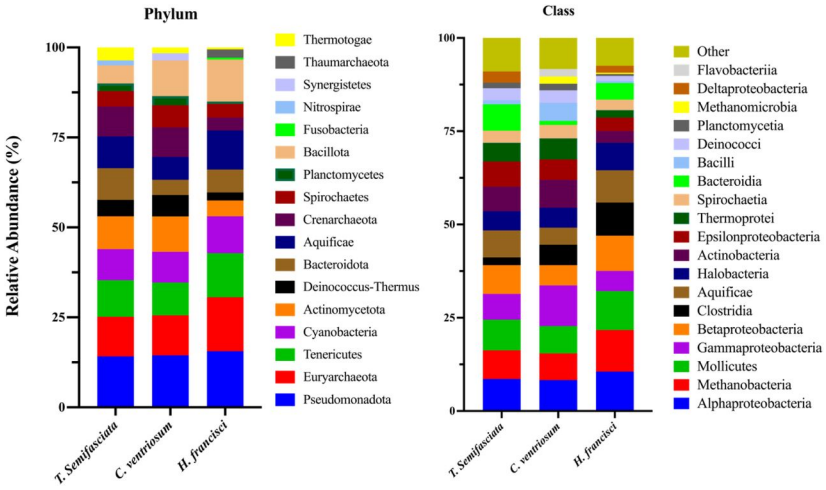


Figure 2: Taxonomic composition of reads from metagenomics sequences from Elasmobranch epidermal microbiomes. Left) Relative abundance of microbial phyla identified three benthic shark species. Right) Top 20 class in ascending order based on average by shark species.

The Kruskal-Wallis H test revealed these variations across species to be significant ($p < 0.001$) between *H. francisci* and both *C. ventriosum* and *T. semifasciata* for the relative abundance of Pseudomonadota and Bacillota. Dissimilarities continued across lower taxonomic levels (all p-values were Bonferroni-corrected). At class taxonomic level, the microbial compositions of microbiomes associated with each benthic shark species exhibited significant differences ($p < 0.001$) for two taxa: GammaPseudomonadota and Methanobacteria. Significant differences ($p < 0.001$) between *H. francisci* and *C. ventriosum* included the bacterial classes Actinomycetota, Mollicutes, Aquificae, and BetaPseudomonadota, while differences between *C. ventriosum* and *T. Semifasciata* were observed for BetaPseudomonadota. At genus level, several microbes unambiguously varied between the shark species (Figure 3). For example, the *Bacteriovorax* genus was only present in *T. semifasciata* ($2.49 \pm 2.15\%$), while both *Coprococcus* and *Ehrlichia* absent only in *T. semifasciata* epidermal microbiomes. Similarly, *Staphylococcus* was not measured in *H. francisci* epidermal microbiomes.

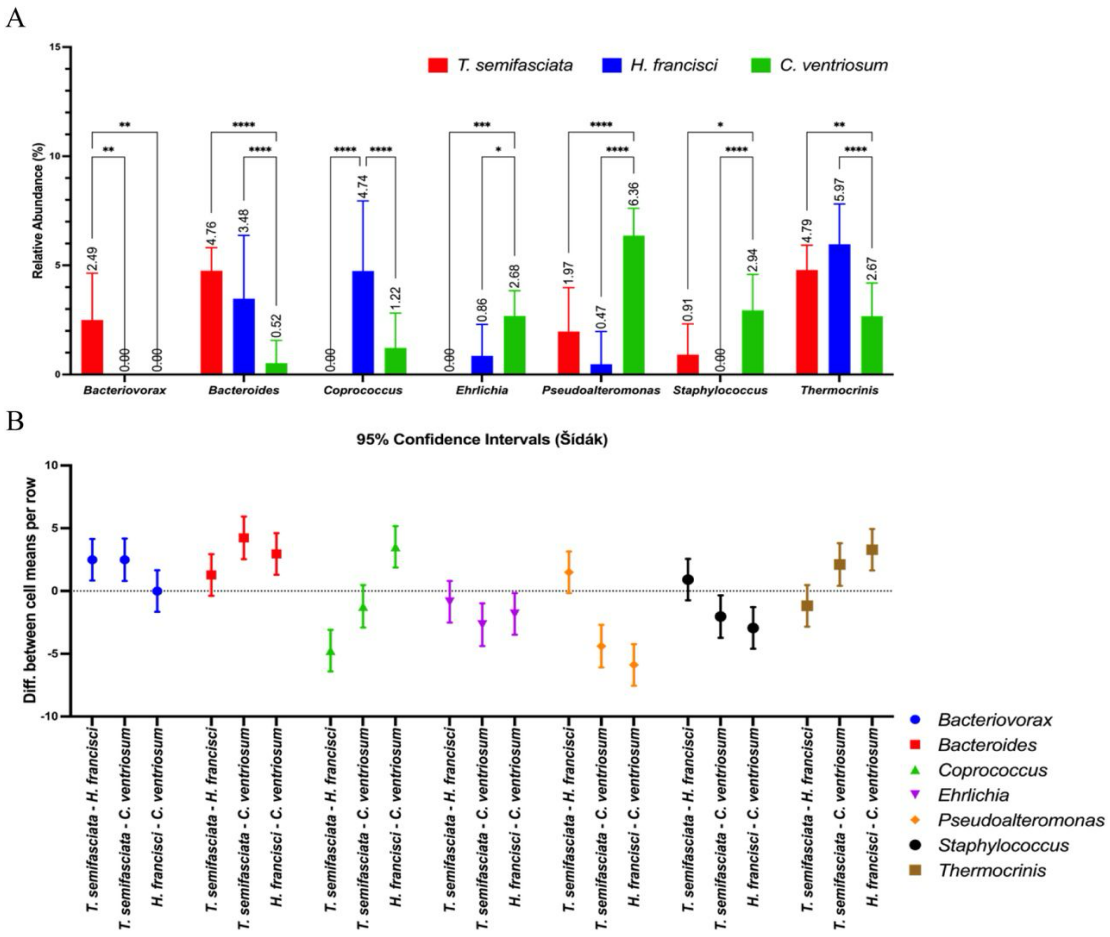


Figure 3: Significant contributors to epidermal microbiomes at A) genus level for associated microbiomes with B) corresponding 95 % confidence intervals.

Pairwise analyses were performed using the Tukey-Kramer post-hoc test at order taxonomic level. The mean abundance order Aquificales on *C. ventriosum* was significantly higher than on both *H. francisci* ($p < 0.001$) and *T. semifasciata* ($p < 0.01$). In contrast, for Alteromonadales, the mean abundance in *C. ventriosum* was significantly lower than in both *H. francisci* ($p < 0.001$) and *T. semifasciata* ($p < 0.001$). For Campylobacterales, *H. francisci* showed a significantly higher mean abundance compared to both *C. ventriosum* ($p < 0.001$) and *T. semifasciata* ($p < 0.001$). Last, for the microbial order Clostridiales, *C. ventriosum* had a higher mean abundance than *H. francisci* ($p < 0.001$), whereas *H. francisci* showed a lower mean abundance than *T. semifasciata* ($p < 0.001$).

We tested the similarity between and within the groups and identified the key microbes contributing to the dissimilarities between shark species. Comparisons of taxonomic community overlap (SIMPER analysis: 100 - dissimilarity index) within epidermal microbiomes associated with *T. semifasciata*, *H. francisci*, and *C. ventriosum* at family taxonomic level revealed *C. ventriosum* microbiomes to have higher similarity within the sample group (91.9) than those belonging to both *T. semifasciata* (90.4) and *H. francisci* (78.4). The SIMPER analysis for abundance data at genus level reported a dissimilarity coefficient of 62.4 between *C. ventriosum* and *H. francisci*, 58.4 between *T. semifasciata* and *C. ventriosum*, and 53.3 between *T. semifasciata* and *H. francisci*. The most important contributors to the epidermal microbiome dissimilarities between each group varied; microbes belonging to the Bacillota phylum were the top contributors to the differences between *T. semifasciata* and *H. francisci,* Methanobacteria were the most influential contributors to the dissimilarity between *H. francisci* and *C. ventriosum*, and Bacteroidia-encompassing microbes impacted the difference between *T. semifasciata* and *C. ventriosum* (Table 3). In pairwise comparisons between *T. semifasciata* and *H. francisci*, the *Coprococcus* genus was found to contribute the most to the dissimilarity, with a contribution of 4.45 %. This genus was present in T. *semifasciata* but absent in *H. francisci*. Other significant contributors included *Methanothermobacter* (3.69 %) and *Methanobacterium* (3.0 %), with the former being absent in *H. francisci*. When comparing *H. francisci* and *C. ventriosum*, *Methanothermobacter* emerged as the top contributor to the dissimilarity, with a contribution of 6.42 %, as it was more abundant in *C. ventriosum* than in *H. francisci* metagenomes. In the comparison of *T. semifasciata* and *C. ventriosum*,

Methanothermobacter again contributed the most to the dissimilarity (5.61 %), being more abundant in *T. Semifasciata*.

Table 3: Pairwise comparison and resulting percent contribution of genera driving differences between epidermal microbiomes associated with each host, determined by SIMPER analysis.

Host Shark Species	Genus	Contribution (%)	Average Relative Abundance	
			T. semifasciata	*H. francisci*
T. semifasciata vs *H. francisci*	*Coprococcus*	4.45	0.0	4.74
	Methanothermobacter	3.69	0.0	3.93
	Methanobacterium	3.0	1.16	3.44
	Haloquadratum	3.01	3.27	5.32
	Jonesia	2.91	4.6	2.01
	Caldivirga	2.78	2.97	0.0
			C. ventriosum	*H. francisci*
H. francisci vs *C. ventriosum*	*Methanothermobacter*	6.42	9.63	4.26
	Methanobacterium	3.19	4.74	1.22
	Coprococcus	3.36	5.18	1.56
	Thermocrinis	2.76	5.97	2.67
	Bacteroides	2.68	3.48	0.52
	Haloquadratum	2.63	5.32	2.67
	Pseudoalteromonas	2.62	0.23	3.5
	Tremblaya	2.5	6.08	3.09
			T. semifasciata	*C. ventriosum*
T. semifasciata vs *C. ventriosum*	*Methanothermobacter*	5.61	4.61	1.3
	Bacteroides	3.62	4.76	0.52
	Methanobacterium	2.35	0.0	2.86
	Pseudoalteromonas	2.45	3.36	1.56
	Ehrlichia	2.29	0.0	2.68
	Caldivirga	2.25	2.97	1.09

We performed PERMANOVA tests on the microbial compositions of each shark to determine if significant differences were present between the epidermal microbiomes associated with each host species. The main PERMANOVA tests identified significant differences in the proportional abundances at family and genus levels (PERMANOVA: family, pseudo-$F_{df = 2, 28}$ =7.65, P(perm) = 0.001; genus, pseudo-$F_{df = 2, 28}$ = 6.79, P(perm) = 0.001), while pairwise PERMANOVA tests outlined significant differences (P(perm) ≤

0.002) between the epidermal microbiome belonging to each species (Table 4) at each taxonomic level (order, family, and genus).

Table 4: Summary of pairwise PERMANOVA results for epidermal microbiome compositions between Elasmobranch species at order, family, and genus level (999 permutations).

	Order Level		Family Level		Genera Level	
Host Factors, Taxa	t(test)	*P*-(perm)	t(test)	*P*-(perm)	t(test)	*P*-(perm)
T. semifasciata vs *H. francisci*	2.26	0.002	2.34	0.002	2.34	0.001
T. semifasciata vs *C. ventriosum*	2.93	0.001	2.55	0.001	2.76	0.001
C. ventriosum vs *H. francisci*	3.11	0.001	3.52	0.001	2.93	0.001

To clarify the relationships among the epidermal microbiomes of different host species, hierarchical clustering was performed at the genus level for each sample. Using the Bray-Curtis similarity index, the microbial genera within the epidermal microbiomes formed distinct clusters, each corresponding to a unique host species (Figure 4A). An nMDS plot showed further clustering of the metagenomes by host species with a stress value under the acceptable threshold (0.13), suggesting the differences between host species are significant influencers over microbial community structure (Figure 4B). Finally, Spearman's rank correlation coefficients, based on microbial genera, demonstrated stronger pairwise relationships between *T. semifasciata* and *C. ventriosum* (0.63), than between *H. francisci* (0.56), and between *H. francisci* and *C. ventriosum* (0.61; Figure 4).

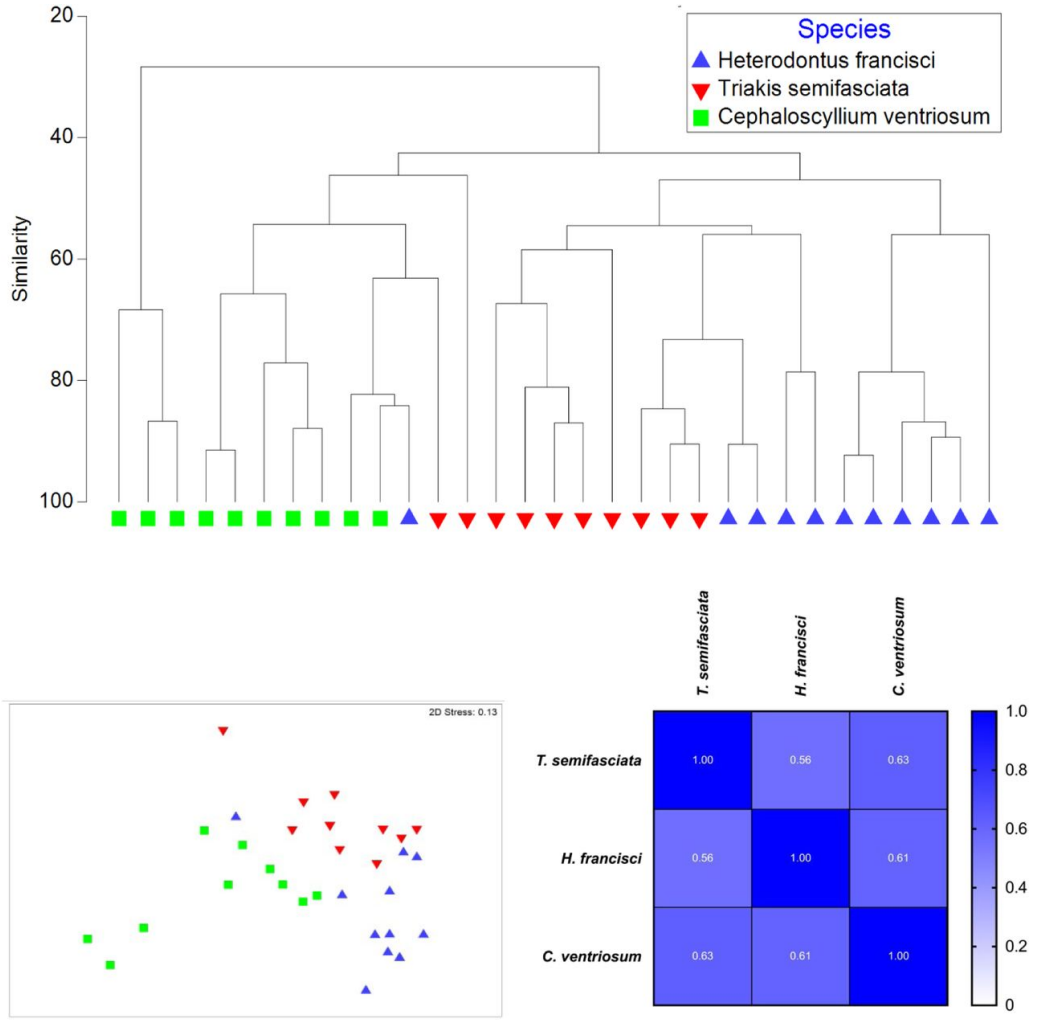


Figure 4: For *Heterodontus francisci*, *Triakis semifasciata*, and *Cephaloscyllium ventriosum*: A) Hierarchical clustering of epidermal metagenomes based on Bray-Curtis dissimilarities derived from fourth root transformed and standardized abundances at the genus level. B) Centroid nMDS ordination plots illustrating spatial differences among epidermal microbiomes. C) Spearman's correlation matrix showcasing correlation coefficients among microbial genera.

Dermal Denticle Morphology and Proportional Abundance Trends

The three shark species had variable dermal denticle morphology (Figure 5). *T. semifasciata* had overlapping dermal denticles, which were slightly elongated, *H. francisci* had square and crown shape dermal denticle that were evenly spaced, whereas *C. ventriosum*

dermal denticles were highly elongated and widely, but unevenly spaced. Therefore, the most remarkable contrast in the morphology of denticles was the spacing between each scale: *T. semifasciata* had the greatest overlap (-197 ± 61.2 µm) while *H. francisci* had a greater average distance between each placoid (226 ± 15.1 µm) and *C. ventriosum* denticles were arranged with the greatest interdenticle distances (426 ± 41.4 µm; Figure 5).

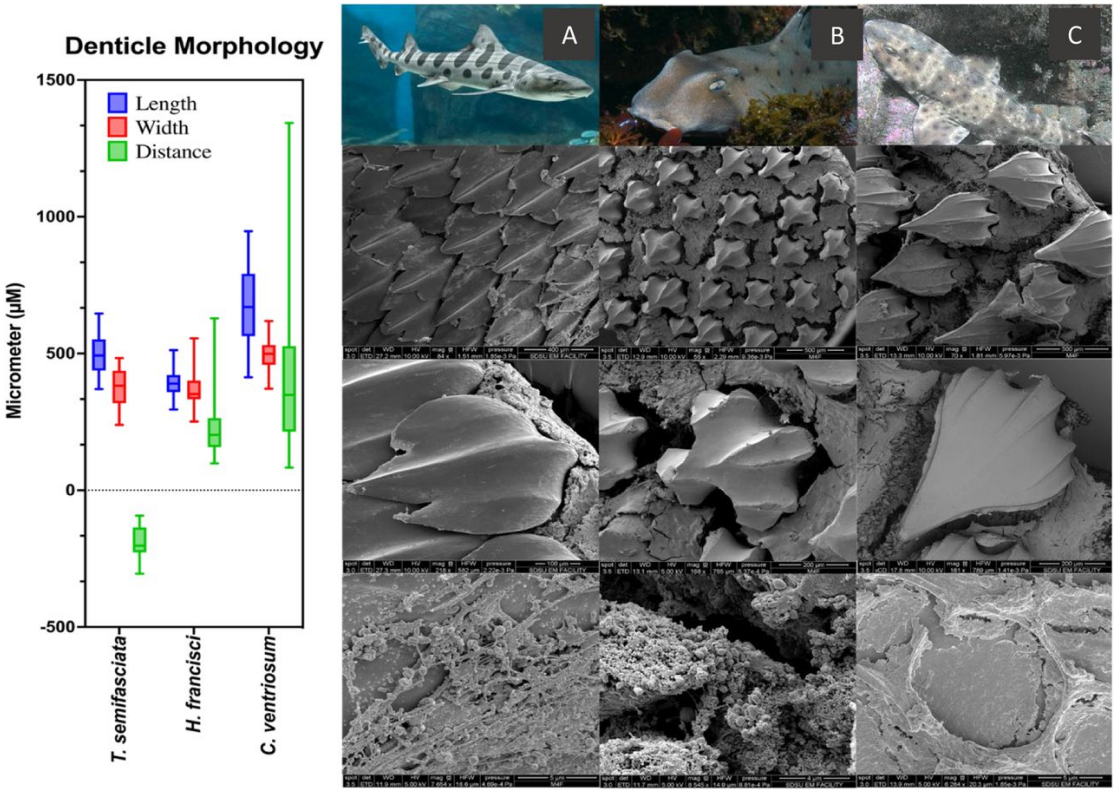


Figure 5: Box plot of denticle morphology depicting denticle length, width, and distance between denticles (left) measured and SEM images (right) at increasing magnification for A) *Triakis semifasciata*, B) Heterodontus francisci and C) Cephaloscyllium ventriosum. Magnification of SEM images in descending order is as follows: 500µm, 200µm, and 5µm.

In our investigation into the relationship between interdenticle distance and microbiome composition, we plotted the distances between denticles against the relative taxonomic abundances of the microbiome for each sampled shark species (Figure 6). Linear regression analysis revealed a moderately positive correlation for *Ehrlichia* ($R^2 = 0.343$,

slope = 0.003) and *Portiera* (R^2 = 0.21, slope = 0.003). In contrast, *Bacteroides* exhibited a negative correlation (R^2 = 0.43, slope = -0.006) with increasing interdenticle distance. Both the positive and negative trends were statistically significant ($p < 0.001$), indicating weak to moderate linear relationships as evidenced by the correlation coefficients. No significant correlation ($p > 0.05$) was observed between interdenticle distance and the relative abundance of *Cyanothece* (R^2 = 0.031, slope = -0.011) when described by simple linear regression. Moreover, the relationship between denticle distance and both *Coprococcus* (R^2 = 0.324) and *Pseudoalteromonas* (R^2 = 0.44) were better described by second order polynomial equations.

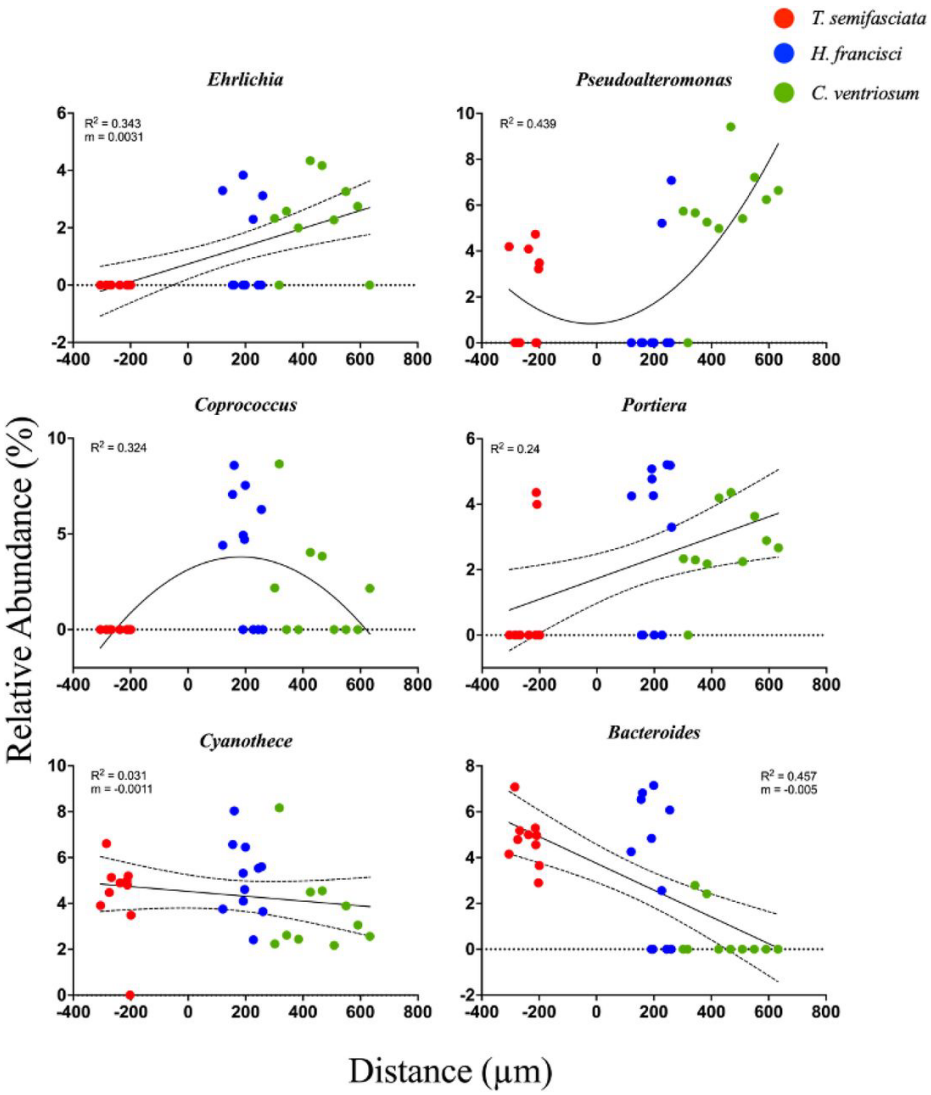


Figure 6: Scatter plots illustrating the correlation between interdenticle distances (x-axis) and microbial relative taxonomic abundance at the genus level (y-axis) for each shark sample. The curves represent the best fit from second-order polynomial regression analyses, and the associated goodness of fit (R^2) and slope (m) values are displayed. The surrounding shaded areas define the confidence intervals for the best-fit curves.

Functional Profiles of Epidermal Microbiomes Across Shark Species

Functional profiles of water-associated microbiomes collected from the captive environment were statistically dissimilar to host microbiomes in PERMANOVA main group tests (PERMANOVA: Genus, Pseudo-$F_{df = 1, 31}$ = 3.21, P(perm) = 0.05) and pairwise

analyses ($p < 0.05$) and are not discussed further. Of the 35 broadest functional genes, pathways involved in carbohydrate metabolism were most abundant (4.83 ± 0.1% S.D.), followed by amino acid synthesis (4.71 ± 0.1%), and protein metabolism (4.5 ± 0.26%). Although no significant difference overall was found between each pair of shark species ($q = 0.85$, $p > 0.5$), utilizing multiple Mann-Whitney tests, we compared the distributions of various functional genes across the three shark species (Table 5). For a more specific comparison, a Kruskal-Wallis H test revealed significant differences across the three species at the more specific functional level II including protein secretion system type 2 ($p = 0.047$), active compounds in metazoan cell defense ($p = 0.023$), general stress response ($p = 0.04$), lysine biosynthesis ($p < 0.001$), once-carbon metabolism ($p < 0.001$), and regulation of virulence ($p = 0.039$; all p-values corrected).

Table 5: Pairwise comparison of mean rank for functional pathways between three shark species: *T. semifasciata* (TS), C. ventriosum (CV), and H. francisci (HF). Table illustrates the p-values, mean ranks, mean rank differences, Mann-Whitney U statistics, and q-values, corrected using the two-stage step-up Benjamini, Kriekger and Yekutieli FDR method.

Functional Pathway	P-value	Mean Rank			Mean Rank Diff.	Mann-Whitney U	q-value
		HF	TS	CV			
Cell Division and Cell Cycle	6.8E-05	6.364		16.1	-9.736	4	6.87E-04
Cell Wall and Capsule	5.5E-04	6.818		15.6	-8.782	9	1.85E-03
DNA Metabolism	7.9E-04	6.909		15.5	-8.591	10	1.99E-03
Dormancy and Sporulation	2.1E-03	7.182		15.2	-8.018	13	3.47E-03
Fatty Acids, Lipids, and Isoprenoids	2.1E-03	7.182		15.2	-8.018	13	3.47E-03
Iron acquisition and metabolism	2.8E-03	7.27		15.1	-7.827	14	3.98E-03
Membrane Transport	8.0E-03	7.64		14.7	-7.064	18	9.45E-03
Miscellaneous	2.8E-03	14.7		6.9	7.827	14	3.98E-03
Motility and Chemotaxis	2.1E-03	14.8		6.8	8.018	13	3.47E-03
Nucleosides and Nucleotides	2.6E-04	15.34		6.2	9.164	7	1.03E-03
Phosphorus Metabolism	1.7E-04	15.5		6.1	9.355	6	8.59E-04
Photosynthesis	1.7E-04	15.5		6.1	9.355	6	8.59E-04
Potassium metabolism	4.0E-05	15.7		5.8	9.927	3	6.87E-04
Protein Metabolism	6.8E-05	6.36	16.1		-9.736	4	9.62E-04
Respiration	3.8E-04	6.73	15.7		-8.973	8	1.54E-03
Secondary Metabolism	2.6E-04	15.4	6.2		9.164	7	1.44E-03
Stress Response	3.8E-04	15.3	6.3		8.973	8	1.54E-03
Sulfur Metabolism	2.8E-03	7.3	15.1		-7.827	14	8.68E-03
Virulence, Disease and Defense	2.8E-03	7.3	15.1		-7.827	14	8.68E-03
Central metabolism	1.1E-05		5.5	15.5	-10	0	3.72E-04

To investigate the differences between the functional profiles of the metagenomes, we performed a SIMPER analysis on the sequenced genes at subsystem level II. Despite our focus on characterizing distinctions, we observed a high similarity between each species (SIMPER analysis: 100 - dissimilarity index), indicating a lack of pronounced differences at this level of analysis. Once again, the *T. semifasciata* group was most similar to *C. ventriosum* (93), while *H. francisci* was more dissimilar to both *C. ventriosum* (86) and *T. semifasciata* (85.3).

The functional gene potential of the microbiomes was significantly distinct across the three species (PERMANOVA, pseudo-$F_{df=2,15}$ = 2.47, $p < 0.001$; Table 6) at the broadest metabolic level. The difference between the three species was also detected when analyzing variations of more specific including SEED subsystem level II pathways (pseudo-$F_{df=2,15}$ = 2.11, $p < 0.05$) and SEED subsystem level 3 (pseudo-$F_{df=2,15}$= 2.32, $p < 0.05$) metabolic pathways. Pairwise PERMANOVA also tests revealed consistently significant variations across species at each functional level (Table 7). However, no singular functional genes at level II subsystems differed between *T. semifasciata* and *H. Francisci*.

Table 6: Summary of PERMANOVA and PERMDISP main test results across benthic shark epidermal microbiome functional subsystem levels I, II, and III (999 permutations).

	PERMANOVA					PERMDISP	
Gene Function: Level I	df	SS	MS	Pseudo-*F*	*P*-(perm)	*F*-value	*P*-(perm)
Benthic Sharks	2	14.4	4.82	5.75	**0.001**	6.56	**0.039**
Res	28	11.7	0.837				
Total	30	26.2					
Gene Function: Level II							
Benthic Sharks	2	47.1	23.6	3.22	**0.001**	10.8	**0.007**
Res	28	102.5	7.32				
Total	30	149.6					
Gene Function: Level III							
Benthic Sharks	2	329.6	109.9	2.65	**0.005**	18.3	**0.004**
Res	28	580.7	41.5				
Total	30	910.3					

df = degrees of freedom, SS = sum of squares, MS = mean sum of squares.

Table 7: Summary of Pairwise PERMANOVA results between benthic shark species at functional subsystem levels I, II, and III (999 permutations).

Host Factors	Level I		Level II		Level III	
	t(test)	*P*-(perm)	*t*(test)	*P*-(perm)	*t*(test)	*P*-(perm)
T. semifasciata vs *H. francisci*	2.11	**0.002**	2.16	**0.001**	1.35	**0.001**
T. semifasciata vs C. ventriosum	1.72	**0.001**	1.78	**0.009**	1.72	**0.011**
C. ventriosum vs *H. francisci*	2.07	**0.001**	2.01	**0.001**	1.31	**0.005**

In our exploration of the linear relationship between interdenticle distance and microbiome functionality, we plotted the distances between denticles against the relative abundance of Level II functional subsystems in the microbiome for each sampled shark species (Figure 7). Linear regression analyses outlined a significantly moderate, negative correlation ($p < 0.001$) between genes encoding for amino sugars ($R^2 = 0.38$, slope = -0.004) and increase distance between denticles. The remainder of the significant relationships, notably those involving genes encoding chemotaxis ($R^2 = 0.18$), regulation of virulence ($R^2 = 0.28$), and desiccation stress ($R^2 = 0.54$), electron-accepting reactions ($R^2 = 0.38$), were more accurately characterized by second order (quadratic) curves, depicting a U-shaped distribution. Functional genes encoding for pyrimidine biosynthesis ($R^2 = 0.38$) was characterized by a bell-shaped curve.

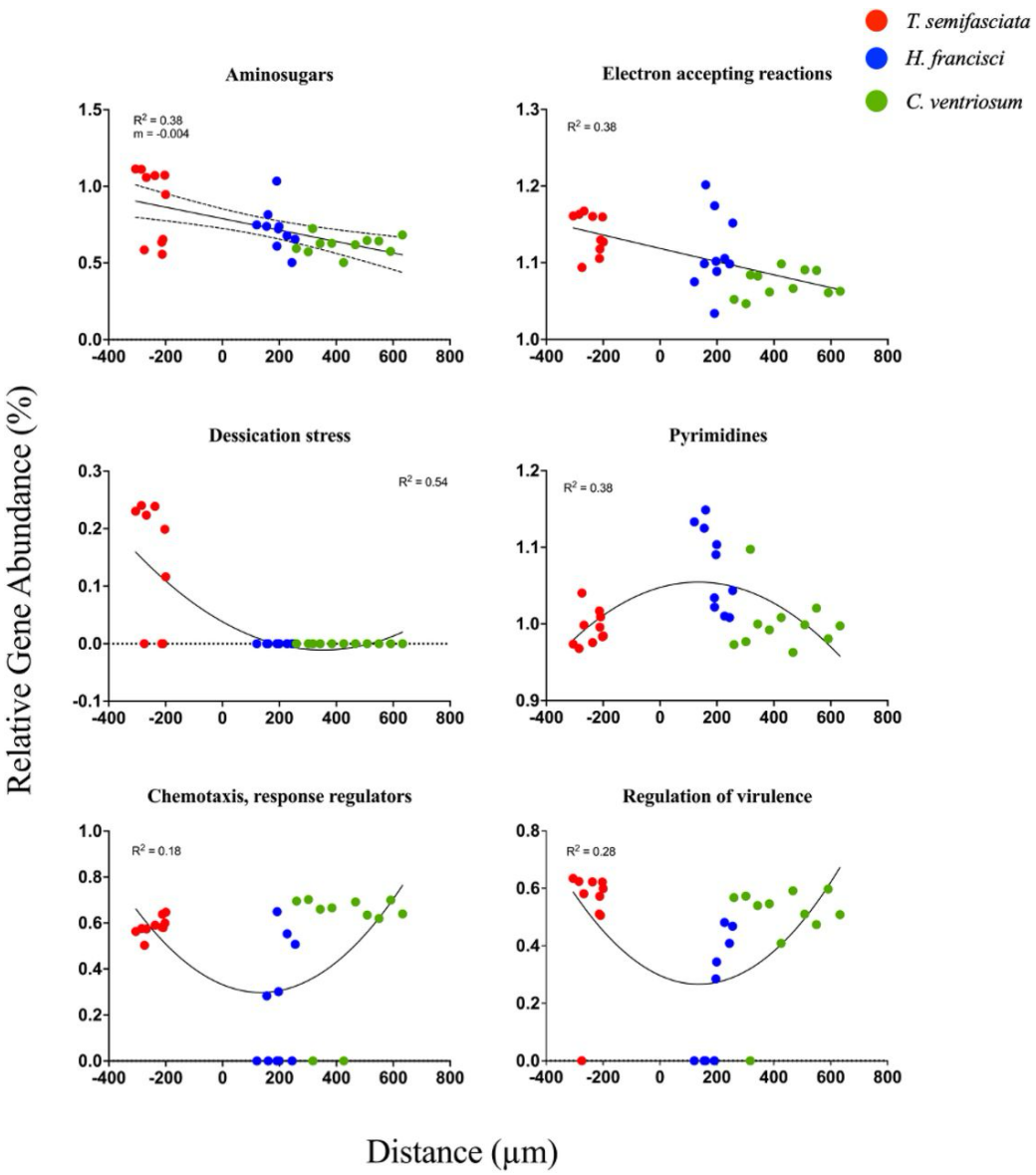


Figure 7: Scatter plots display the relationship between interdenticle distances (x-axis) and microbial relative functional gene abundance at SEED subsystem level 2 (y-axis) for each shark sample. The depicted best-fit lines are derived from both linear and second order polynomial regression analyses, each complete with their respective goodness of fit (R^2) and slope (m).

The comprehensive multiple group comparisons did not uncover discernible linear correlations co-varying with the trajectory of denticle distance. However, these comparisons did provide evidence of host species-specific gene function levels. For example, the genes

associated with desiccation stress were exclusively found in the microbiomes of *T. semifasciata*, with a significant average frequency ($p < 0.05$) of 0.113 ± 0.09 %. Notably elevated levels of genes associated with pyrimidine biosynthesis were discernible in the metagenomes of *C. ventriosum*, with a significant average frequency ($p < 0.001$) of 0.142 ± 0.1 % while a significant reduction ($p < 0.01$) in the levels of genes associated with virulence regulation in *H. francisci* microbiomes (0.17 ± 0.19 %) as compared to those observed in *T. semifasciata* (0.48 ± 0.2 %) and *C. ventriosum* (0.49 ± 0.1 %) was observed. We investigated phylosymbiotic trends in both the taxonomic composition and functional gene profiles of the epidermal microbiomes associated with our three shark species. The theory of phylosymbiosis posits that as the evolutionary distance between host species increases — as determined by differences in the COX1 gene in our study — the dissimilarity of their associated microbiomes should also increase. This divergence can manifest in both the types of microbes (taxonomic dissimilarity) and in the functional genes those microbes carry (functional dissimilarity). Between the three species, *H. francisci* featured the farthest evolutionary distance from *C. ventriosum* and *T. semifasciata*, and we found significant increases in microbiome distance as the evolutionary distance increased ($F_{df = 1,144} = 5.1$, Adj – $R^2 = 0.04$, $p < 0.05$, Figure 8, left), supporting the argument for phylosymbiosis. The gene function comparisons corroborated the phylosymbiotic trends, with significant increase in dissimilarity again ($F_{df = 1,144} = 64.3$, Adj-R2 = 0.3, $p < 0.001$, Figure 8, right).

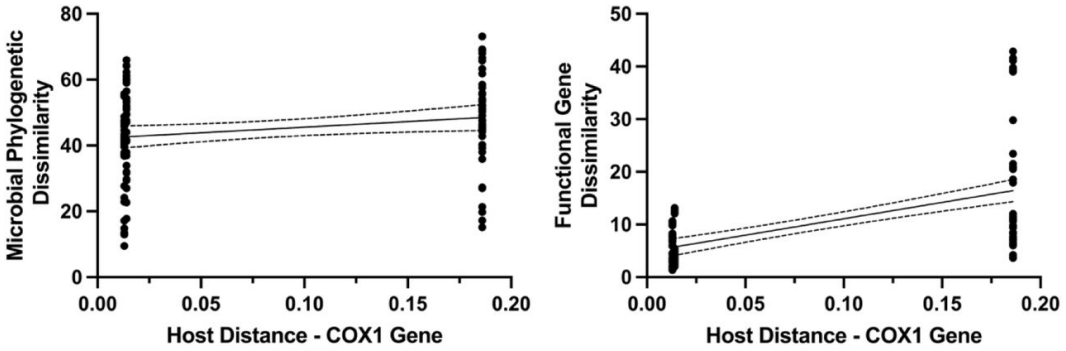


Figure 8: Phylosymbiotic comparison between host genetic divergence (COX1 gene) and epidermal microbiome phylogenetic dissimilarity (left) and gene function dissimilarity (right).

Discussion

Heterodontus francisci, *Triakis semifasciata*, and *Cephaloscyllium ventriosum*, maintain an evolutionary distance of approximately 200 million years, with *T. semifasciata* and *C. ventriosum* belonging to the Carcharhiniform order and *H. francisci* of the Heterodontiformes and the host's phylogenetic lineage shaped the epidermal microbiome, even when these benthic sharks inhabit the same coastal environment. Therefore, the unique morphological and physiological traits of the sharks directly impacted the relationship between host and epidermal microbiomes. Our research clarifies these intricate relationships, demonstrating that each shark species harbors a distinct microbial community characterized by both taxonomic composition and functional gene profiles. Interestingly, these microbiomes are influenced by the specific dermal denticle topography of the host, suggesting an intricate interplay between physical host characteristics and microbial colonization.

We evaluated the impact of host evolution across the sharks and observed a clear grouping of the *C. ventriosum* metagenomes in the plotted dendrogram, while *H. francisci* and *T. semifasciata* samples are more interspersed, suggesting more variability in the

microbial communities of these species. However, consistent with the phylosymbiotic trend *T. semifasciata* and *C. ventriosum* metagenomes are more closely related to each other than *H. francisci* metagenomes. Moreover, metrics outlined by performing SIMPER analysis corroborate a significant co-evolution congruity in the epidermal microbiomes between *C. ventriosum* and *T. semifasciata* shark species. Finally, we found that as the evolutionary distance between host species increased, as measured by differences in the COX1 gene, the dissimilarity of their associated microbiomes also increased. This pattern was observed both in the taxonomic composition and functional gene profiles of the microbiomes, suggesting a deep intertwining of host evolution and microbiome development.

Building on phylosymbiotic trends, we found each shark species to harbor unique microbiome composition. For example, significant variation at phylum level, including differences in Pseudomonadota and Firmicute abundance between the three host species. While the skin of the shark species was dominated by Pseudomonadota, species-specific variations were observed among the Pseudomonadota classes Beta- and Gamma-proteobacteria. These distinctions echo a similar study finding fish, captive dolphins, whales, and killer whales which harbor species-specific microbiomes [42]. Even though each host harbored a species-specific microbiome, we did observe some common features. Most notably, the Pseudoalteromonadaceae family was dominant across all shark hosts, consistent with microbiome surveys of *T. semifasciata* [16]. This family of bacteria is well-known for its crucial roles in biofilm formation and the deterrence of potential microbial predator colonization[43,44]. An equally important contributor to the microbial compositions are the *Sphingomonodacaea* population which, belonging to the Alpha-proteobacteria clade, are key contributors to initial biofilm formation and are able utilize a wide array of organic

compounds[45]. Together, these two major contributors dominate the microbiome of *T. semifasciata*, *H. francisci*, and *C. ventriosum*, and we theorize these phyla represent core contributors and regulators to the microbiome composition of Elasmobranchs, being identified in multiple shark and ray species [5,8,15,19].

We investigated the impact of a host-derived factor, denticle topography, on the microbial compositions across the three species by quantifying the relationship between microbial abundance and interdenticle distance. We postulated that the overarching influence on microbial symbiotic associations across host species would be dictated by host phylogeny, with the effects of denticle topography exerting a selective influence on individual microbial constituents, contingent upon their unique bacterial characteristics, in a case-specific manner and found substantiating evidence for this theory. For example, the *Staphylococcus* genus was significantly more abundant in the metagenomes of *C. ventriosum*, less so in *T. semifasciata*, and completely absent in *H. francisci*. The associative pattern may be dictated by host phylogeny via differences in denticle coverage and subsequent mucus production across the three species. To better understand this phenomena we look to one study in which the attachment of two bacteria, *Escherichia coli* and *Staphylococcus aureus*, displayed varied attachment rates based on the surface of the synthetic models mimicking shark skin[46]. The attachment of *S. aureus* was inhibited on the surfaces resembling the highly structured shark denticle environment. Therefore, *Staphylococcus* associations may be selectively favored by the unique combination of decreased denticle coverage and increased mucus production characteristic of *C. ventriosum*. To build upon this theory, we posit the enhanced denticle overlap and less pronounced riblet elevation in conjunction with minimal but protein-rich mucus of *T. semifasciata* skin,

provides a conducive surface for *Staphylococcus* to colonize[47], while *C. ventriosum* offers the greatest opportunity for providing mucus production as a function of exposed epidermis. Last, the distinct structural environment of *H. francisci's* dermal denticles, characterized by pronounced riblets and troughs, may be less hospitable to *Staphylococcus*, despite the potential assistance provided by mucus produced from a more exposed epidermal environment. Studies outline *Staphylococcus* species, such as *S. aureus*, to be considered common primary pathogens and implicated in wound infections[48–50].

We understand coastal marine environments may serve as reservoirs of antibiotic-resistant strains of *Staphylococcus*, and the increased abundance across shark species may be indicators of increasingly opportunistic pathogenicity of associated microbiomes[51–53]. We observed similar phylosymbiotic trends between *Pseudoalteromonas* abundance and shark hosts, whereby host phylogeny served as a greater predictor of microbial association than respective denticle morphology. The associative behavior of the *Pseudoalteromonas* genus is a key contributor to biofilm regulation due to initial adhesion capabilities, subsequent pathogenic inhibition via the production of antibiofilm molecules[43,54,55], and biosurfactants[56]. While the adhesion behavior of *Pseudoalteromonas* has not been investigated in the context of synthetic shark epidermal models, we can theorize this genus assumes the archetype of prolific biofilm initiator [57] and regulator [58] across all three species. Taken together, our observations underscore the intricate relationship between host denticle topography and microbial taxa, with *Staphylococcus* and *Pseudoalteromonas* serving as illustrative examples of how microbial colonization patterns are influenced by the host's phylogenetic lineage via unique denticle topography.

Building on our observations, we were intrigued by the taxonomic abundances which echoed the denticle morphology. For example, *Ehrlichia*, often associated with pathogenic outcomes, and *Portiera*, known for its symbiotic relationships in insect hosts, both show positive covariance with increasing denticle distance. This association may reflect their adaptability to the physical environment provided by the denticle morphology, yet the specifics of this relationship are not well understood, and further research is warranted. It is therefore interesting to consider the ecological interaction between these bacteria and the composition of the shark denticles: denticle composition offers an ecological niche rich in organic matter including collagen fibers, which provide the scaffolding for the primary dentin mineral Hydroxyapatite[59]. Consequently, the moderate, negative covariance between *Bacteroides* and increasing interdenticle distance proved interesting given the wide distribution of the genus in marine degrading complex biopolymers, such as polysaccharides and proteins, which are integral to biofilm formation and organic carbon cycling[60]. The observed negative correlation between this genus and increasing interdenticle distance suggests that the decrease in denticle density may lead to a reduction in the available organic matter derived from enamel and dentine, substances that *Bacteroides* utilize, and therefore deter *Bacteroides* biofilm formation. Overall, the interplay between denticle morphology and bacterial associations is a complex, multifaceted relationship that extends beyond the confines of physical structure to include factors such as host physiology, including mucus production, and other environmental conditions.

The functional profiles of the epidermal metagenomes showed *T. semifasciata* microbiomes were most similar to *C. ventriosum*, while *H. francisci* was more dissimilar to both *C. ventriosum* and *T. semifasciata*, indicating functional redundancy and co-

evolutionary trends and mirroring of phylogenetic distances between the species. We observed a significant, negative, covariance between genes encoding for both amino sugars and electron-accepting reactions with greater interdenticle distance. The observed relationship suggests that as denticle distance increases, microbes could be adapting their metabolic processes, specifically those related to amino sugar utilization, in response to the physical structure of the epidermis. This adaptation, reminiscent of how marine bacteria modulate their amino sugar production based on environmental cues, could involve an upsurge in the production of glucosamine and galactosamine, two pivotal amino sugars in bacterial physiology prevalent in marine ecosystems[61]. These linear trends establish a direct connection between shark skin morphology and specific functions of the epidermal microbiome. However, we also observed complex interplay between denticle distance and functional profiles of epidermal microbiomes when we mapped curvilinear relationships mirroring host phylogeny more closely than denticle morphology. For example, we observed U-shaped curves for chemotaxis, desiccation stress, and regulation of virulence, and bell curve line fitting pyrimidine biosynthesis, indicating a higher concentration of these genes at both the lower and higher extremes of interdenticle distance. This pattern of gene abundance, shaped by denticle distance, has notable implications for the functional potential of the microbiome, as these genes play critical roles in key microbial processes. Virulence regulation is implicated in disease-causing capabilities in bacteria in response to environmental cues including nutrient concentrations, pH, temperature, and host-derived factors[62]. The diminished representation of virulence regulation-associated genes within the epidermal microbiome associated with *H. francisci* implies heightened microbiome stability; a decreased necessity for bacterial pathogenesis could be indicative of a host-derived

microbial community rather than an environmentally driven one, given habitats are conserved across the three species[63,64].

Further investigation into each bacterial community's tendency to follow or deviate from phylosymbiotic trends yielded insights into potential ecological interactions. For instance, genes related to desiccation stress were uniquely present in the microbiomes of leopard sharks, potentially due to the placement of goblet cells beneath the epidermis, resulting in a reduced mucus layer compared to other taxa. This suggests a heightened reliance on the dermal denticles for protection against desiccation. By highlighting functional gene differences, we further the knowledge about the influence of denticle distance for each shark host on associated bacterial populations. For example, the high denticle overlap covaried with elevated relative levels of genes encoding chemotaxis and flagellar movement suggests motile bacteria utilize the consistent and expansive area on or underneath the overlapping denticles instead of relying on the fluidics of the aqueous environment. The increased motility facilitated by flagella in the metagenomes can be theorized to allow bacteria to infiltrate and navigate biofilms, leading to a greater overall area of adhesion[65]. The relationship between bacterial motility and denticle overlap is invites varying interpretations. Although bacterial motility is not integral to biofilm initiation[66], consistent surfaces presented by high overlap may facilitate biofilm formation, which requires swimming and twitching motility to navigate. Conversely, as denticle spacing increases, the necessity for enhanced bacterial motility could arise to navigate the intricate, aqueous environment, rather than relying on passive, random movement[65,67]. Further research is required to clarify the discriminating forces and reconcile these perspectives.

Previous efforts to understand these forces have already yielded insightful results. For instance, research conducted by Doane *et al.,* which investigated the microbiomes of Elasmobranchs of *Rhincodon typus*, *T. semifasciata*, and *A. vulpinus*, found no phylosymbiotic trends were observed within their functions [19]. However, we detected phylosymbiotic patterns between the benthic species in this study both for microbial compositions and functional profile similarity. While we cannot completely dismiss the role of denticle distance or mucosal production as selective mechanisms, we posit the measured phylogenetic distance between *H. francisci* and both *T. semifasciata* and *C. ventriosum* is greater than that between *R. typus, T. semifasciata* and *A. vulpinus* and therefore, the pressure exerted by evolutionary distance is more evident. This theory is strengthened when considering sampling location as a driver of microbiome composition. If the environment exerted a greater pressure, we would expect to observe higher similarity between the benthic shark, and yet our findings revealed significant dissimilarities both between and within the groups.

Caveats

Coupling core microbiomes of healthy animals with host-derived factors of microbiota recruitment will aid in the development of reliable biomarkers of shark ecology. However, although we have identified correlations between denticle topography and microbiome composition, our study design does not allow us to establish causality. Also, due to the high complexity of abiotic factors (e.g., water temperature, pH, salinity, and dissolved oxygen), and future threats of increased ocean temperatures, further research is needed to test the influence of environmental variables on the ability of marine hosts to

recruit and retain epidermal microbes under extreme temperature shifts in controlled settings.

Conclusion

In an exploration of the intricate relationship between host physiological characteristics and microbial community structures, we hypothesized the unique dermal denticles morphology of each shark species would parallel the influence of host phylogeny on the composition and structure of their corresponding epidermal microbiomes. Preliminary observations of epidermal microbiome taxonomic compositions and functional potentials differed between *T. semifasciata*, *H. francisci*, and *C. ventriosum* irrespective of the hosts sharing a captive environment. However, while our results reveal a compelling concordance between the diversity of epidermal microbiomes and host phylogeny among the examined shark species, we also observed consistent, yet weak, linear relationships with respect to denticle distance, a trait that does not follow the same phylogenetic pattern. This suggests a potential complex interplay between host evolutionary history and specific morphological traits in shaping the shark epidermal microbiome. The results of this study support the notion of phylosymbiosis, while suggesting denticle morphology provides a template for the assembly of microbial communities on shark skin.

Materials and Methods

Epidermal microbiomes of captive *T. semifasciata* (n = 4), *H. francisci* (n = 3), and *C. ventriosum* (n = 10) were sampled in the summer of 2018 at the Birch Aquarium at Scripps Institution of Oceanography in La Jolla, California. In the summer of 2019, captive *T. semifasciata* (n = 6) were again sampled at the Birch Aquarium. Finally, in the summer of 2020, the epidermal microbiome of *H. francisci* (n = 8) were sampled in captivity at the

National Oceanic and Atmospheric Administration (NOAA) in La Jolla, California. For all sampling events, captive sharks were immobilized in a sling for consistent collection of epidermal microbiomes located between the pectoral and dorsal fins above the lateral line on the left flank of each shark. Epidermal microbiomes were collected using a blunt, closed-circuit syringe prefilled with 100 kDa filtered seawater to flush the epidermis and displace microbes [19,25,26]. Approximately 200 mL of captured microbes were then collected on a 0.22 μm sterivex, with one sterivex per individual., Water-associated microbial communities were collected using bulk water samples where approximately 60 L of tank water were simultaneously collected and first filtered through a nylon mesh sieve (200 μm pore size) to remove unwanted debris and eukaryotic organisms and second, concentrated using tangential flow filtration (100 kDa; [27,28] to produce ~500 mL of tank water. The resulting concentration of tank water was filtered using a 0.22 μm sterivex.

Microbial cells anchored in sterivex filters were lysed by incubating the filters at 37 °C and 25 μL of proteinase K/SDS solution and resulting free DNA was extracted and purified using the Macherey-Nagel NucleoSpin Tissue Kit. The eluted DNA was prepared for shotgun metagenomic library sequencing using the Swift 2S Plus Kit (Swift Biosciences) and sequenced using an Illumina MiSeq sequencer. Samples were run in tandem using DNA barcoding throughout several sequencing runs as performed in previous studies [26,27,29]. Resulting reads were processed for quality to remove artificial duplicates: reads with greater than 10 unknown nucleotides (n), and reads fewer than 60 base pairs (bp) in length via Prinseq++ [30]. High quality, paired end reads were annotated via the Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST; Keegan, Glass, and Meyer 2016) online database. MG-RAST calls taxonomic and functional gene assignments using BLAST

comparisons to the National Center for Biotechnology (NCBI) and SEED genome databases [32]. Sequencing annotations were conducted using the following parameters: e-value $>10^{-5}$, 70 % identity, and > 60 bp alignment length.

Skin punches of 6 mm diameter were obtained from the dorsal flank regions of *H. francisci*, *T. semifasciata* and *C. ventriosum* sharks as metagenomic samples were collected. Biopsy specimen included the top layer of denticles and underlying dermal layers. Samples were rapidly frozen in liquid nitrogen (LN_2) upon collection and maintained at cryogenic temperatures until fixed. Then, a 2.0 % glutaraldehyde ($C_5H_8O_2$; Electron Microscopy Sciences, Hatfield, PA cat # 16100) and 0.1 M cacodylate buffer ($C_6H_{12}AsNO_2$) was prepared fresh and the frozen skin punches dropped into the room temperature fixative. Samples were stored in fix at 4°C until prepared for scanning electron microscopy (SEM) analysis.

For SEM, tissues pieces were washed three times with 0.1 M cacodylate buffer to remove residual fix. They were further post-fixed in 1.0 % osmium tetroxide (OsO_4; Electron Microscopy Sciences; Hatfield, PA cat #19150) in 0.1 M cacodylate buffer for 60 minutes at room temperature. Samples were dehydrated through a standard ethanol series of 30 %, 50 %, 75 %, two times 95 % and two times 100 % for 15 minutes each. Tissues pieces were critical point dried using liquid carbon dioxide (CO_2) using a Samdri 790 CPD (Rockville, MD, USA). After drying, shark skin pieces were mounted with denticles facing up on 12mm Al stubs and double sticky carbon tape. The stubs were coated with ~6nm platinum before viewing on FEI Quanta 450 SEM (Quanta 450, Hillsboro, OR USA) at 5-10kV.

For each shark species, the dimensions of the dermal denticles were measured. Dermal denticles were imaged at 100x magnification with a minimum of five fields per shark. On each image, five random denticles were selected and the widest and longest point of the denticle was measured. In addition, the distance to the nearest denticle was measured to provide an estimate of the inter-denticle distance.

Statistical Analyses

To study the impact of host phylogeny on the microbiomes of three captive shark species, we used several statistical analyses used historically in microbiome research[27,33,34]; first, to address the potential influence of rare taxa on the results, we fourth root transformed the data, followed by standardization [28], which is preferred to rarefaction [35,36]. To assess the alpha-diversity of the microbial communities, we used Margalef's (*d*), Pielou's (*J'*), and Inverse Simpson's ($1/\lambda$) indices to measure richness, evenness, and diversity, respectively [37–39]. We tested the beta-diversity of the microbiomes by first comparing the microbiomes of sharks to the water column and second comparing between shark species using permutation multivariate analysis of variance (PERMANOVA). The PERMANOVA ran with 999 random permutations per analysis. To identify the similarities and differences between the groups, we calculated similarity percentage breakdowns (SIMPER)[40]. Mean rank comparisons were performed using the multiple comparison Friedman test, and to control the false discovery rate, the two-stage step-up method of Benamini, Krieger and Yekutieli was used. We conducted both PERMANOVA and non-parametric Kruskal-Wallis H tests on the relative abundance of microbial taxa from the levels of order to genus to examine changes in the microbiome's taxonomy belonging to each host species. These tests were chosen as both do not assume a normal distribution and are appropriate for comparing three

or more unrelated groups. Following community-level analyses, post-hoc analyses were performed to interpret the pairwise differences between the three shark species. In cases where the Kruskal-Wallis H test identified significant differences, the Tukey-Kramer post hoc test was applied to conduct pairwise comparisons between the groups. This post hoc test adjusts for multiple comparisons, reducing the likelihood of Type I errors. Last, a Bonferroni correction was applied to further control the family-wise error rate, adjusting the significance level to account for the multiple hypotheses tested. An alpha of 0.05 was used as the significance level for statistical tests.

To visualize the associations between metagenomes belonging to different but closely related species we generated non-metric multidimensional scaling (nMDS) derived from Bray-Curtis matrices [41]. We chose an nMDS for its rank-based method of representing complex and non-linear relationships between multiple variable data. The Bray-Curtis dissimilarity was used as it effectively measures differences in community composition, accounting for the presence, absence, and abundance of species. A PERMDISP analysis was used to test for differences in group dispersion or homogeneity of the multivariate variations.

We simultaneously analyzed the abundance of genes in the microbiome as a proxy for gene expression. To assess the functional potential of the metagenomes of captive *H. francisci*, *T. semifasciata* and *C. ventriosum* sharks, we again used a PERMANOVA analysis. We also conducted an ANOVA with a post hoc Tukey test to identify differences in metabolism, which were visualized using the Statistical Analysis of Metagenomic Profiles (STAMP; v2.1.3; https://beikolab.cs.dal.,ca/software/STAMP) software. All statistical analyses were performed using Primer-e package 7 (v7.0.2; accessed on 28 January 2022; www.primer-e.com/permanova.html) with the PERMANOVA+ add on, STAMP, and

GraphPad PRISM 9 (v9.1.2; https://www.graphpad.com), and R studio. All graphs were generated using GraphPad PRISM 9. The SEED's Subsystem Annotation was used to categorize the functional pathways into a hierarchical structure, ranging from broad metabolic pathways (Subsystem Level 1) to increasingly specific gene functions (Subsystem Levels II & III). This allowed us to map key biochemical functions to their parent pathways.

The correlation between microbial family relative abundance and the distance between shark denticles was examined using a regression model. To assess the strength and significance of the correlation, a scatter plot of the two variables was generated for each genus, along with respective p-value. The least squares regression line was superimposed on the scatter plot and the R-squared value was calculated, indicating how well the line fit the data.

To test whether skin microbiome composition was linked with host phylogeny, we calculated host distance by aligning the cytochrome c oxidase I (COX1) gene of each species using Clustal Omega on the EMBL-EBI server using default parameters. COX1 genes were downloaded from NCBI and used because it represents the only host gene publicly available for host phylogenetic comparison. We determined the relationship of host distance to microbiome similarity using linear modeling (lm; R)[19].

Institutional Review Board Statement

Animal handling and ethics were reviewed at San Diego State University through IACUC under permit APF #14–05-011D, APF #17–11-010D, APF # 18–05-007D, approved May 24th, 2018. Sampling was conducted under state permit SCP #12847 and SCP #9893 from the California Department of Fish and Wildlife.

Data Availability

All data and sequences were deposited in the NCBI Sequence Read Archive database.

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Conserve, cull, compromise — the white shark conundrum

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Abstract

This review examines the complex relationship between white sharks (*Carcharodon carcharias*) and humans in marine environments, focusing on human-wildlife conflicts and associated implications for white shark management. Incidents involving shark bites are rare but often tragic and traumatic, sparking significant public concern and leading to controversial culling practices that raise further ethical and ecological questions. We explore the conserve, cull or compromise response measures on shark populations, while emphasising the necessity for adaptive, but alternative management approaches to culling.

Case studies are used to demonstrate co-existence strategies, including community education, improved beach safety protocols and habitat protection. Furthermore, we discuss future directions for research and policy, advocating for collaborative frameworks that prioritise human safety without compromising shark conservation. Ultimately, this review highlights the importance of reducing human-wildlife conflict through different scenarios of conserve, cull, or compromise to foster a more balanced and positive relationship between humans and white sharks, ensuring both can persist and co-exist safely in their shared environment.

Introduction

The white shark (*Carcharodon carcharias*), often revered and feared as one of the ocean's most majestic predators, has long been the subject of fascination and controversy. This enigmatic species faces multiple challenges globally. It is listed as Vulnerable to extinction by the International Union for the Conservation of Nature's Red List of Threatened Species (hereinafter IUCN Red List) (Rigby et al., 2022), and is protected within most jurisdictions where it occurs. The reputation of this species as a formidable predator has made it an iconic symbol of oceanic ecosystems, but the white shark has also become the focal point for debates surrounding ecology, conservation and human-wildlife conflict (Laroche et al., 2007; Nel & Peschak, 2006). The white shark conundrum, or 'wicked problem' (Niella et al., 2021), encompasses the struggle to balance the conservation of the species with the safety and concerns of coastal communities that recreate in their local marine environment. Understanding the multifaceted issues surrounding the white shark is

crucial for evidence-based decision-making aimed at promoting human safety as well as the persistence of healthy white shark populations.

This review delves into the white shark conundrum, providing differing and often controversial views on the diverse facets of this complex issue and the implications it holds for marine conservation and human society. With a growing need to understand the scale of impact white sharks have in ecosystems, their protection under various legal frameworks and strategies for reducing human-shark conflict, a comprehensive examination of the subject is vital., While we have some understanding around the importance of white sharks in our oceans (Dedman et al., 2024), there continues to be a need for knowledge on the significance of white sharks not just as predators, but as a higher order species that influences the structure and function of marine ecosystems. We combine the expertise and experience of 68 scientists, managers, industry representatives and other relevant stakeholders brought together during the first international white shark conference in 13 years, White Sharks Global (Port Lincoln, Australia, 2023), to synthesise current information and views related to the white shark conundrum. We delve into each of the three main pathways discussed by the general public and among stakeholders to manage white sharks; (i) protect this threatened and protected species (*conserve*), (ii) exclude or kill animals from popular swimming and surfing areas (*cull*), or (iii) concede that a combination of options will be possible in the Anthropocene (*compromise*). We conclude that stand-alone policies of conserving or culling white sharks are overly simplistic for this multifaceted and complex issue and that strategies aimed at reaching a compromise and that are adaptive are necessary for meeting the dual objectives of white shark conservation and human safety.

White sharks in marine ecosystems

White sharks are large (up to 6 m total length and 2,000 kg weight) marine predators distributed globally through temperate, sub-tropical and tropical coastal and oceanic regions (Bonfil et al., 2005; Compagno, 2002; Domeier, 2012; Duffy, 2016). White sharks occupy high trophic levels (Compagno, 2002; Hussey et al., 2015) and influence marine ecosystems through consumptive and non-consumptive effects (Beltran et al., 2021; Hammerschlag et al., 2022, 2017; Dedman et al., 2024), exerting top-down control and regulating lower trophic levels (De Vos et al., 2015a; Moxley et al., 2019). The broad-scale movements of white sharks can be influenced by a range of abiotic drivers, including sea surface temperature (e.g., Lipscombe et al., 2023; Jorgensen et al., 2010; White et al., 2019), lunar phase (Gooden et al., 2025a, Weltz et al., 2013; Werry et al., 2012), wind direction (Gooden et al., 2025a), and biotic factors such as prey availability (Bruce et al., 2006; Heithaus & Vaudo, 2004; Kock et al., 2018), primary productivity (Lee et al., 2021; Shaw et al., 2021), and predatory pressure from larger species such as killer whales (*Orcinus orca*; Jorgensen et al., 2019; Towner et al., 2024, 2022, 2023; Reeves et al., 2025). In the northeast Pacific, juvenile white sharks are appearing more frequently at higher latitudes (Tanaka et al., 2021), and in the northwest Atlantic, may also be increasing at higher latitudes off Atlantic Canada (Bastien et al., 2020). Along the South African coast, a recent eastward shift in distribution has been proposed (Bowlby et al., 2023), though this claim has been contested (Gennari et al., 2024) and subsequently addressed in a response (Bowlby et al., 2024). Nonetheless, the shift of white sharks away from previous aggregation sites (e.g., Jorgensen et al., 2019; Towner et al., 2022) can have a cascading effect on local ecosystems, releasing other top predators (e.g., broadnose sevengill shark (*Notorynchus cepedianus*), and mesopredators, e.g., bronze whaler (*Carcharhinus brachyurus*) to fill the ecological gap resulting from

white shark displacement (Hammerschlag et al., 2019; Dedman et al., 2024). Further, the departure of white sharks from marine communities can change the physiology and behaviour of prey species, (e.g. Cape fur seals, *Arctocephalus pusillus pusillus*; Hammerschlag et al., 2017; Hammerschlag et al., 2022; De Vos et al., 2015a, b). Increasing anthropogenic pressures from overfishing, urbanisation, and climate change are influencing prey availability and distribution, in addition to predator-prey dynamics (Dedman et al., 2024; Gooden et al., 2025b), which has potential to displace white sharks, initiating ecological cascades (Hammerschlag et al., 2019; Towner et al., 2022). The range of factors driving white shark range shifts and the subsequent impacts of their absence from specific ecosystem components complicates population recovery, coastal management and the mitigation of negative shark-human interactions (Lagabrielle et al., 2018).

Humans in marine ecosystems

Human interaction with the oceans is highly complex and holds great socio-economic and cultural importance at the individual, community and global scale (Barange et al., 2010). The ocean is a source of food, energy, recreation, and income through commercial and recreational activities (e.g., fishing, diving, wildlife tourism, deep-sea mining and drilling, wind and wave energy generation). Human use of the marine environment, particularly extractive processes, carries implications for the stability of ecosystems, the fitness of marine species and the livelihood of people who engage with the ocean. The perception, value, and use of the ocean, varies among societies demographically, regionally, economically and culturally (Barange et al, 2010). However, human influence on ocean ecosystems has increased substantially throughout the Anthropocene (McCauley et al., 2015; O'Hara et al., 2021). We humans could therefore be considered apex predators in

terms of our ability to exert pressure on marine environments, directly (e.g. extractive industries such as fishing, mining and energy production; O'Hara et al., 2021), passively (e.g. surfing, diving, and swimming), and indirectly (e.g. activities such as wildlife tourism activities that mediate species interactions; Tisdell & Wilson, 2004).

Large, charismatic megafauna, including the white shark, have long been the subject of human intrigue, with archaeological records and anecdotal evidence indicating that sharks have been a significant symbol and resource for cultures around the world (Skubel et al., 2019). This long-held fascination has developed through multifaceted interactions that can be beneficial and harmful for both humans and sharks. Within these interactions, responsibly managed wildlife tourism offers a valuable opportunity to learn about the ecological value of various species and provides inspiring experiences through the direct observation of animals in their natural habitats (Apps et al., 2018, 2016). However, negative interactions with sharks can also occur during recreational (e.g. surfing, swimming, diving) or occupational (e.g. commercial diving) activities (Chapman & McPhee, 2016; Cliff and Dudley, 2011; West, 2011), leading to injuries, deaths, and impacts on livelihoods and communities, including economic loss from reduced tourism, as well as psychological impacts through post-traumatic stress at both the individual and community level (Curtis et al., 2012; Engelbrecht et al., 2017; Taylor et al., 2019). Hence, the co-existence between white sharks and people remains more nuanced than often portrayed, requiring a balanced and informed approach (Ferretti et al., 2015). Recreational and occupational ocean users (e.g. surfers, swimmers, divers) have minimal impacts on white shark behaviour, but are potentially most vulnerable to negative shark interactions due to disproportionately long periods of time spent in the ocean compared to the rest of the human population. Shark-human interactions often

draw significant media and public attention due to the potentially severe impacts of these encounters and because white sharks, like other large predators (e.g., bears, crocodiles, wolves, etc.), are unpredictable, dangerous and sometimes unavoidable (Jacobs & Vaske, 2019; Johansson et al., 2012; Magnuson, 1987; Peace, 2015). Indeed, we cannot control or modify an animal's natural behaviours. Thus, a multitude of measures have been developed to protect humans from shark interactions (Curtis et al., 2012; McPhee & Blount, 2015; McPhee et al., 2021). However, we can control how we respond to shark-human interactions. Human-wildlife conflict is, in reality, less about conflict between humans and wildlife and more about the disagreements among people over how to address the issue (Redpath et al., 2015). Despite the low probability of occurrence and risks of shark bites being overstated, a single negative event has the potential to amplify voices disproportionately, exerting a significant influence on decisions that can greatly impact white sharks and potentially wider ecosystem processes. The crucial role of humans in the contemporary structure of marine ecosystems must be examined with the knowledge that humans and white sharks often overlap in time and space (e.g. Engelbrecht et al., 2017; Winton et al., 2021; Rex et al., 2023). The management of white sharks goes beyond cultural and legislative boundaries. Thus, a collaborative and multi-faceted approach is required to manage shark-human interactions and anthropogenic ocean use.

‘Conservation’ efforts

White sharks are long-lived, slow growing and late maturing, resulting in low reproductive rates (Bruce, 2008; Calliet et al., 1985; Francis, 1996; Natanson & Skomal, 2015). This combination of life history traits makes white shark populations vulnerable to depletion and extirpation. White shark populations have suffered major declines since the

1950s due to overexploitation by commercial fisheries, trophy hunting for jaws or teeth and lethal shark control programs (Dewar et al., 2013; Ellis & McCosker, 1991; Rigby et al., 2022). Globally, white shark populations have declined by up to 70 – 90% (e.g. in the Northwest Atlantic Ocean, Mediterranean Sea, and Australia's east coast; Curtis et al., 2014; Moro et al., 2020, Rigby et al., 2022; Roff et al., 2018), leading, not only to concerns for the conservation of the species, but also to the health and structure of marine ecosystems (Dedman et al., 2024; Hammerschlag et al., 2022; Hammerschlag et al., 2019). Although strategic fisheries management and conservation programs (Dewar et al., 2013) have led to the recovery of white shark populations in some regions of the world (e.g. Northeast Pacific—Dewar et al., 2013; Northwest Atlantic—Curtis et al., 2014), the species remains listed as Vulnerable by the IUCN Red List (Rigby et al., 2022). The documented population declines and inherent vulnerability of the species have resulted in legislated protection across a number of jurisdictions, including Australia, Canada, United States, Mexico, and South Africa (Malcolm et al., 2001; Bruce et al., 2018), as well as the listing of white sharks under the Convention on International Trade in Endangered Species (CITES – Appendix I + II) and the Convention on Migratory Species (CMS – Appendix I + II). In spite of these concerted conservation efforts, areas remain where poor enforcement and monitoring render white shark populations particularly susceptible to illegal, unregulated, or unreported capture (Madigan et al., 2021; Milazzo et al., 2021). Additionally, many populations are data deficient, e.g., in the Northwest Pacific (e.g. Russia, Korea, Japan, China, Taiwan, Philippines, Vietnam; Christiansen et al., 2014), Indonesia (Dharmadi et al., 2015), and to a lesser degree the Mediterranean basin (Moro et al., 2020, Ferretti et al., 2024). Even where protection exists, bycatch in various coastal and pelagic fisheries occurs (Curtis et al., 2014;

Lyons et al., 2013; Rigby et al., 2022). While white sharks appear to have a high post-release survival rate in some net fisheries (Benson et al., 2018; Lyons et al., 2013), immature individuals are particularly susceptible to fishing activities (Lowe et al., 2012; Oñate-González et al., 2017). White sharks also remain vulnerable to threats associated with shark control programs (Dudley, 1997; Gibbs et al., 2020; Kock et al., 2022; Niella et al., 2021), fisheries pressure (Lowe et al., 2012), and shifts in short and long-term climate (Tanaka et al., 2021), particularly for populations whose distribution extends beyond protected areas.

Conservation strategies used to reduce white shark mortality and ensure population recovery vary in scale and effectiveness. In fisheries, they include prohibitions on retention (Curtis et al., 2014), bycatch mitigation (Benson et al., 2018; Dewar et al., 2013; Klimley, 1985; Lyons et al., 2013; Oñate-González et al., 2017), improvements in monitoring and reporting of white sharks catch and restrictions placed on gear, such as gillnets and purse seines (Sacchi, 2021). In addition, there have been several advances in the development of non-lethal shark-bite mitigation programs (Engelbrecht et al., 2017; Kock et al., 2012; Tate et al., 2021), deterrent measures (Huveneers et al., 2018; 2013, McPhee et al., 2021; Riley et al., 2022), management of wildlife tourism operations (Gooden et al., 2024; Meyer et al., 2021; Niella et al., 2023) and no-take protected areas (Albano et al., 2021; Dulvy, 2006), which prioritise white shark conservation principles. The listing of white sharks on national acts and international treaties, such as CITES and national recovery plans under the auspices of the International Plan of Action for the Conservation and Management of Sharks (FAO, 1999), further solidifies intentions to conserve and protect this species. While white shark populations are the focus of extensive conservation efforts, it is increasingly essential to assess and monitor population sizes, demographics, connectivity between populations and

shifting distributions due to climate change. Such information is critical for estimating extinction risk, population recovery, and assessing the efficacy of management strategies to protect white sharks and mitigate human-shark conflict.

At present, white shark population sizes across the world's major ocean basins remain uncertain despite varied small-scale research attempts (Andreotti et al., 2016; Burgess et al., 2014; Chapple et al., 2011; Hillary et al., 2018; Kanive et al., 2023; Towner et al., 2013; Winton et al., 2021). Furthermore, regional estimates are often debated (Burgess et al., 2014) and are heavily focused on immature sharks (neonate, juvenile and sub-adults), with a lack of information on adults. Additionally, most efforts have focused on estimating census sizes (total numbers of animals; *N*c), while efforts to characterise effective population sizes (numbers of reproductive adults; *N*e) are less common. The rapid declines observed in the mid to late 20th century have pushed some white shark populations to critical levels, where recovery has relied on a small number of reproductive adults. Evidence from South Africa estimates the white shark population being at critical levels (*Ne* between 247 and 487 and high levels of inbreeding in the juvenile and subadult parts of the population: Andreotti et al., 2016). Although this has been debated (Irion et al., 2017; Bowlby et al., 2022), it underscores the challenges of accurately assessing the population status of white sharks. Despite ongoing studies, the total population remains relatively small (most likely <2,000 individuals). Bowlby et al., (2022) modelled the annual catch of white sharks in the KwaZulu-Natal shark control program under different removal scenarios, and concluded that this systematic culling alone is likely to restrict the species' chances of recovery in the future. Similarly, population genetic research has pointed to high levels of relatedness in juvenile and subadult Australian white sharks and estimated the overall effective population

size (*N*e) to be less than 300 individuals (Blower et al., 2025; Clark et al., 2025). These findings raise significant conservation concerns, highlighting risks of potential inbreeding, and reductions in population fitness and resilience. Consequently, while population recovery has been observed in some regions of the world (based on *N*c estimates; Dewar et al., 2013), the genetic health and diversity of recovering populations may be compromised and warrants careful attention to inform conservation management.

Future conservation of white shark populations also relies on maintaining and improving educational messages and communicating conservation priorities to the public, key stakeholders, and management organisations. Public perception is an important consideration that can and should influence policies (Cullen-Knox et al., 2017; Neff 2012). For example, a series of shark bites by white sharks in Cape Town, South Africa, in the early 2000s led to public calls for the expansion of the KwaZulu-Natal shark (lethal) control program into Cape Town waters (Nel & Peschak, 2006). In response, local government initiated a public participation and expert engagement process to evaluate all available options. This process ultimately led to government support for Shark Spotters, a grassroots initiative that provided continuous shark surveillance and was seen as a compromise between a conservation-only approach (doing nothing) and culling. Over time, the program expanded to include research and education, further enhancing its role in shark safety and conservation (Kock et al., 2012).

Other initiatives, such as the Atlantic White Shark Conservancy's Gills Club (organisation for children, especially 8–13 years old girls), have had a positive influence on the public perception of shark conservation by fostering STEM-based education, arranging direct interactions between community members and marine scientists, and providing

opportunities for members to engage in local projects relating to sharks and ocean conservation. Similarly, education and community engagement are among the highest priorities in the New South Wales shark management program (Martin et al., 2022; Simmons et al., 2021; Simmons & Mehmet, 2018). These examples demonstrate the various strategies to effectively communicate the need for shark conservation and to include all stakeholders in the refinement and implementation of conservation initiatives.

'Culling' white sharks: controversies and consequences

While there has been increasing support for non-lethal approaches to shark bite mitigation (Curtis et al., 2012; Martin et al., 2022; Simmons et al., 2021; Simmons & Mehmet, 2018), lethal measures have been used globally (e.g., in Australia, South Africa, Reunion Island, and New Caledonia), with the first government-led large-scale program initiated off Sydney (Australia) in 1937. Culling is the archetype of lethal measures, and while some measures that do not aim to kill sharks can lead to sharks (or other species) dying, lethal measures are typically synonymised with culling programs. In this context, culling is defined as the 'killing of animals with a view of controlling numbers', which can take many forms and be implemented on a variety of spatial and temporal scales (Table 1). For example, culling of white sharks ranges from public-led, often haphazard, localised attempts to capture the shark responsible for a bite in the aftermath of an incident, to long-term, government-coordinated programs deploying standardised fishing gear at specific sites (Cliff & Dudley, 2011; Curtis et al., 2012; Losen, 2023; Wetherbee et al., 1994), some of which have significantly reduced populations to such levels which consequently reduced human-shark interactions by up to 90% (Dudley, 1997). In some regions, shark control programs using gill nets and baited drumlines with the aim to cull sharks have been in place

for decades (e.g., the South African and Australian shark control programs). They have, however, become increasingly controversial in light of global declines of large sharks and other marine megafauna (Pacoureau et al., 2021; Juan-Jordá et al., 2022), the recognition of their importance in healthy oceans (Dedman et al., 2024) and growing recognition of some programs inefficacy with no measurable benefits (Cliff & Dudley 2011; Dulvy et al., 2014; Wetherbee et al., 1994).

White sharks are particularly susceptible to population declines from fishing due to their life history traits (Pacoureau et al., 2021; Rigby et al., 2022), facilitating rapid reduction in population size during culling programs. However, due to the range of factors influencing shark-human interactions and the difficulty estimating white shark population sizes, whether culling is effective remains uncertain (Carlson et al., 2019; Curtis et al., 2012; Gibbs et al., 2020; Neff, 2015; Neff & Yang, 2013). Furthermore, while long-term culling programmes may result in perceived increased safety for beachgoers, this is often undermined by a lack of public understanding of the manner in which this is achieved. In a survey conducted at South African beaches with both lethal and non-lethal shark safety measures in place, only 8% of respondents understood that shark nets reduce risk by catching and killing sharks, with the majority incorrectly assuming that they form physical barriers that prevent sharks from entering the bathing area (Sheridan et al., 2021). Culling predators has often been an unsuccessful strategy to reduce human-wildlife conflicts. In Central Karoo, South Africa, culling black-backed jackals to protect farmers was counterproductive and resulted in increased livestock losses (Nattrass et al., 2019). In Slovenia, grey wolves were culled between 1995–2009 to reduce depredation on livestock, but had no measurable effect on subsequent depredation rates (Krofel et al., 2011). Culling

grey and harbor seals in Iceland to protect fish stocks between 1982 and 2002 also had no measurable effect on fish stocks in the area during that time (Bowen & Lidgard, 2013). Meanwhile, in Hawaii, an expensive multi-year culling program for tiger sharks was ineffective at reducing the number of shark bites, which, in fact, increased during and after the program was implemented (Wetherbee et al., 1994). Culling can also escalate the disparate opinions on the conflict between humans and wildlife, and should be approached with caution when proposed as a risk mitigation strategy (Nunny, 2020).

Culling programs operate on the assumption that lowering shark densities inherently reduces the risk to humans at specific geographic locations (Lemahieu et al., 2017). Given the life history characteristics of white sharks and consequent vulnerability to even low levels of mortality (Bowlby et al., 2022), targeted fishing programs can certainly reduce the abundance of white sharks (Roff et al., 2018). Shark control programs are also one of the activities (alongside game fishing and bycatch from commercial fishing) responsible for declining white shark populations (Rigby et al., 2022). Following the implementation of the shark control programs in South Africa and around Sydney (Australia), catch rates of large sharks, including white sharks, declined and coincided with a reduction in the frequency of shark bites (Dudley 1997, Gibbs et al., 2020). Recent increases in the number of shark bites in regions where populations of large sharks are recovering in response to conservation and management measures may seem to support the premise behind culling programs (Winton et al., 2021), but could also be reflective of human population growth in coastal areas, as well as the increased popularity of recreational water sports (Ferretti et al., 2015, Gibbs et al., 2020). One of the challenges with culling programs is understanding the number and biological class (e.g. age, sex, size) of sharks that need to be culled. While the argument can

be made that removing any sharks reduces risk, a large proportion of the white shark population would likely need to be culled to ensure that the risk of shark-human interactions is substantially reduced. This is because they (all life stages to some degree) directly spend a large proportion of time in coastal waters in habitats where humans undertake recreational and commercial activities. However, any culling contradicts the protected status of the species in most countries where the sharks naturally occur, and referrals or special permits would need to be obtained to allow culling programs.

One alternative to large-scale or long-term culling programs is the targeted killing/removal of individual sharks (Clua et al., 2020; Clua & Linnell, 2019). In most cases, identifying and locating the individual shark responsible for an attack is challenging, but advances in genomic techniques are improving this likelihood. Genetic profiling on samples from the bite area (e.g. saliva) can already be used to identify the species responsible for a bite (Lafferty et al., 2018; Mukherjee et al., 2007; Martin et al., 2024; van Rooyen et al., 2021) and can be extended to identify individual sharks and inform selective removal of individuals (Clua et al., 2022). However, implementing this in a timely manner and in many countries would be challenging in practice. There are rare occasions when the shark responsible for a fatal incident has been reliably identified and remains in the area, potentially enabling targeted removal of that individual., For example, a three-metre bull shark was caught the day after a 22-year-old surfer was fatally bitten in Noosa (Australia) in 1961 and the man's leg was retrieved from the shark’s stomach (Noosa News, 2019). More recently, remains were recovered from a tiger shark after it bit a tourist at a popular beach in the Red Sea (Egypt) in 2023. A necropsy of the shark indicated the animal was emaciated and in poor condition, potentially motivating the incident.

Targeting individual sharks might serve multiple purposes; it may provide closure to the family of the victim bitten by the shark, provide insight into the cause of the bite and it could reduce further risk as this particular shark will no longer be able to bite humans. The main arguments often used to rationalise the targeting of individual sharks as a mitigation measure are that 1) some individual sharks might be naturally, or have become, more likely to bite humans (e.g., bold individuals, sharks in poor conditions, or sharks that have developed behavioural changes following previous interactions with humans) and that 2) removing any shark reduces the likelihood of bites occurring. There are rare instances when individual sharks have been responsible for several bites or have been considered 'problem' individuals (Clua et al., 2024). It should be acknowledged that the targeted removal of sharks suspected or confirmed as being responsible for a bite, may or may not necessarily reduce future risks.

Culling programs often have the support of local and vocal community groups, particularly in communities that have been affected by sharks and sectors aligned with beach-related tourism and aquatic recreation activities, as well as some commercial and recreational fishing organisations. Conversely, other community groups, and perhaps counterintuitively many survivors of serious shark bites, are influential advocates for shark conservation and strongly opposed to shark culling. The significant bycatch associated with lethal measures is often used to criticise culling programs, as gill net and drum lines are known to capture non-target species including teleost fish, marine reptiles, cetaceans, and non-target Elasmobranchs (Curtis et al., 2012), some of which are recognised as being threatened and/or protected (Atkins et al., 2013; Broadhurst & Cullis, 2020). Consequently, debates can become highly politicised during election campaigns, especially in the wake of

an incident (Niella et al., 2021). Adapting mitigation measures (e.g., lethal - e.g. euthanise to non-lethal drumlines - e.g. catch and release) may be a more palatable solution than eliminating culling completely, as those responsible for human safety are often hesitant to end long-term shark culling programs for fear of being liable for future shark bites (McCagh et al., 2015; Niella et al., 2021).

It is also important to recognise that not all shark-bite mitigation programs are necessarily 'culling' programs. For example, the New South Wales Shark Meshing Program's goal, when introduced in 1937, was to cull any and all sharks. However, over time, the operation and management of the program have changed such that there are now only three target species (i.e., white, tiger and bull sharks), which are tagged and released if found alive (Reid et al., 2011; Tate et al., 2021). Similar changes are being trialled in Queensland's 2021–2025 Shark Management Plan following the decision in 2019 of the Administrative Appeals Tribunal that ended the deliberate culling of sharks in the Great Barrier Reef Marine Park. Yet, target sharks and non-target species die in gill nets every year, which remains controversial due to ethical issues and negative conservation outcomes (Gray & Gray, 2017; Pepin-Neff & Wynter, 2018). It should also be recognised that culling programs often contribute to scientific studies through the collection of biological samples and sharing of large datasets. For example, the shark control programs in Kwa-Zulu Natal (South Africa) and eastern Australia have contributed to and led hundreds of scientific publications (Cliff & Dudley, 2011; Dudley & Simpfendorfer, 2006; Heupel & Simpfendorfer, 2010; Dalton et al., 2024).

The objectives underlying culling practices are frequently a subject of debate. Are these measures intended to capture and eliminate individual sharks following an incident, or

do they aim to maintain the population of a species below a specific threshold to achieve reduced or zero interactions with humans? Determining this threshold and effectively communicating it to the public during culling operations is crucial., Society must establish clear metrics for evaluating the success of modern culling programs, such as aiming for zero or near-zero human-shark interactions in targeted areas, and determining the appropriate course of action if these objectives are not met. Unfortunately, a scientifically robust determination of whether shark bite mitigation measures are effective is difficult as shark bites occur infrequently and at spatiotemporally stochastic levels (Huveneers et al., 2024). Should governments persist with existing management strategies, or should they adapt their approaches? An increasing body of evidence points to the latter and the inefficacy of some current culling approaches. For example, negative human-shark interactions at Cid Harbour, Australia, prompted an immediate culling response, yet subsequent shark bites continued. Furthermore, shark bites continue to occur at netted beaches in New South Wales, Australia, albeit these levels have not been compared to the growth of numbers of people using the waterways. These examples highlight the complexity of the issue and the need for a thorough presentation of the facts for assessing the efficacy of culling as a strategy for reducing shark bite risk.

Table 1. Examples of shark culling strategies used by management authorities and locations where they have been implemented.

Type of culling	**Methods and scale**	**Examples**	**References**
Direct response to shark attack	Targeted fishing in an area, over days, weeks, or months	Reunion Island	Losen (2023)
Long-term bather protection	Drumlines or gill nets, strategically placed on		Cliff and Dudley (2011)

Type of culling	Methods and scale	Examples	References
	popular beaches, permanent or seasonal	Kwa-Zulu Natal, South Africa; Queensland, Australia; Hawaii, USA	Wetherbee et al., (1994)
Opening of shark fishing specifically to reduce population numbers	Opening commercial fishing with gill nets or drumlines, permanent or seasonal	Reunion Island, proposed in Western Australia	Guyomard et al., (2019)
Recreational fishing	Trophy tournaments aimed for the capture of large sharks	Florida	Shiffman & Hammerschlag (2014) Gallagher et al., (2017)
Targeting of 'problem' individuals	Opportunistic when shark remains in the area following a bite	Egypt	Clua et al., (2020) Clua and Linnell (2019)

'Compromise' in white shark management

The premise of 'compromise' in this context is that most stakeholders and policy makers believe that mitigating shark bites is an appropriate approach to protect ocean users, but that it should be implemented using measures that have minimal negative impacts on sharks and other marine life. Policy makers accept that neither pro-conservation nor pro-cull members of the community will be entirely pleased with the outcome, but that both parties are more likely to accept the decision when their views are considered as part of a transparent and consultative management process (Figure 1). Policies governing shark bite responses are a top-down driver of white shark management but are influenced by public

perception and media attention in the wake of shark bites. While some studies have emphasised that public attitudes towards sharks may be independent of the occurrence of shark-human interactions (Neff & Yang, 2013), public apprehension resulting from shark bite incidents have prompted calls for policy action (Neff & Yang, 2013; Pepin-Neff & Wynter, 2018). It could be argued that management and response decisions should not be made immediately following a serious or fatal shark bite, as rational thought and decision-making could be compromised due the emotional consequences of the incident for all involved. Minimising conflict between white sharks and humans is, therefore, the central theme to effectively achieving compromise and the subsequent use of policies and management that functionally offers some, but not absolute protection for ocean users, while meeting conservation objectives.

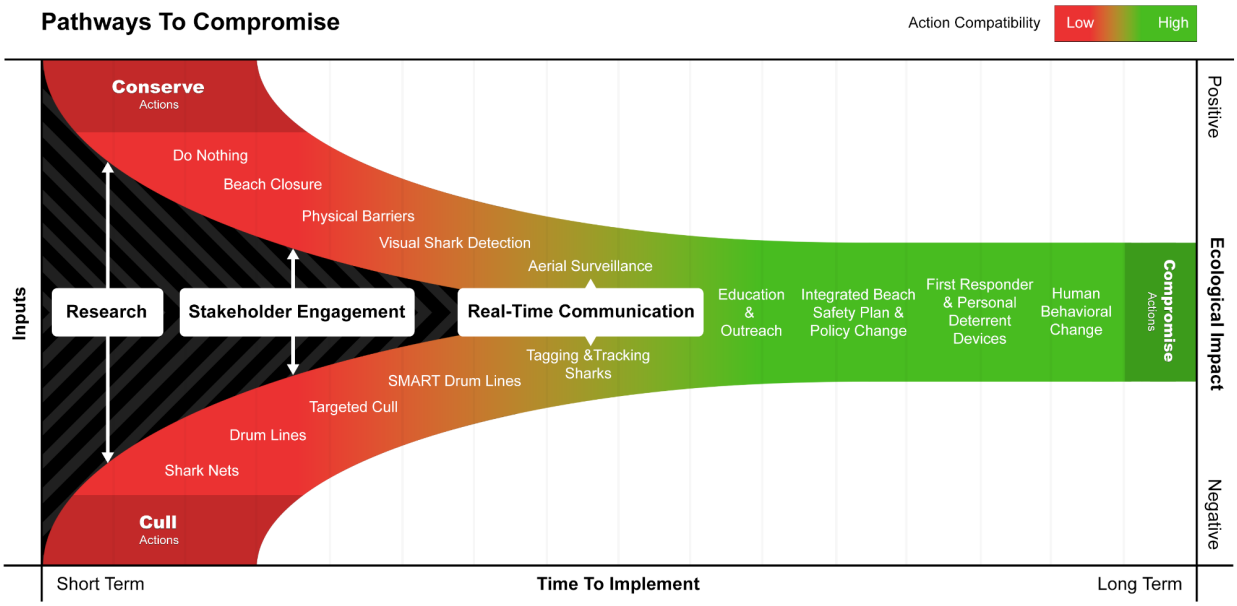

Figure 1. Various management pathways to achieve compromise between conservation-only and culling-only strategies for sharks.

There are many examples of effectively implementing ‘compromise’ programs for a range of potentially dangerous terrestrial wildlife globally, highlighting that 'compromise' human-wildlife conflict policy can work in practice (Figure 2). Increasingly, examples of compromise solutions/policy are emerging for white sharks, driven through a combination of community (i.e., bottom-up) and authority-led (i.e., top-down) interventions. Community-led shark safety programmes are often critical contributors to the success of achieving compromise solutions and driving policy change during periods of “shark crisis”. During the early 2000s in Cape Town, South Africa, a spate of fatal and non-fatal shark-bite incidents at popular recreational beaches motivated the local community to develop an immediate response to reduce shark bite risk, in the absence of a clear authority-led strategy (Kock et al., 2012; Oelofse & Kamp, 2006). Local businesses provided funding for trained observers (known as spotters), to conduct continuous visual surveillance, using the area's elevated coastal topography (*c.a.* > 40 m), to detect white sharks at two high-conflict beaches. With a confirmed white shark sighting close to shore, the spotter would communicate the potential risk to the beach using a radio, triggering a shark alarm to alert and temporarily evacuate water-users. This informal, real-time, early-warning initiative proved to be an effective non-lethal solution, reducing shark-human spatial overlap and restoring recreational water user confidence in Cape Town (Oelofse & Kamp, 2006).

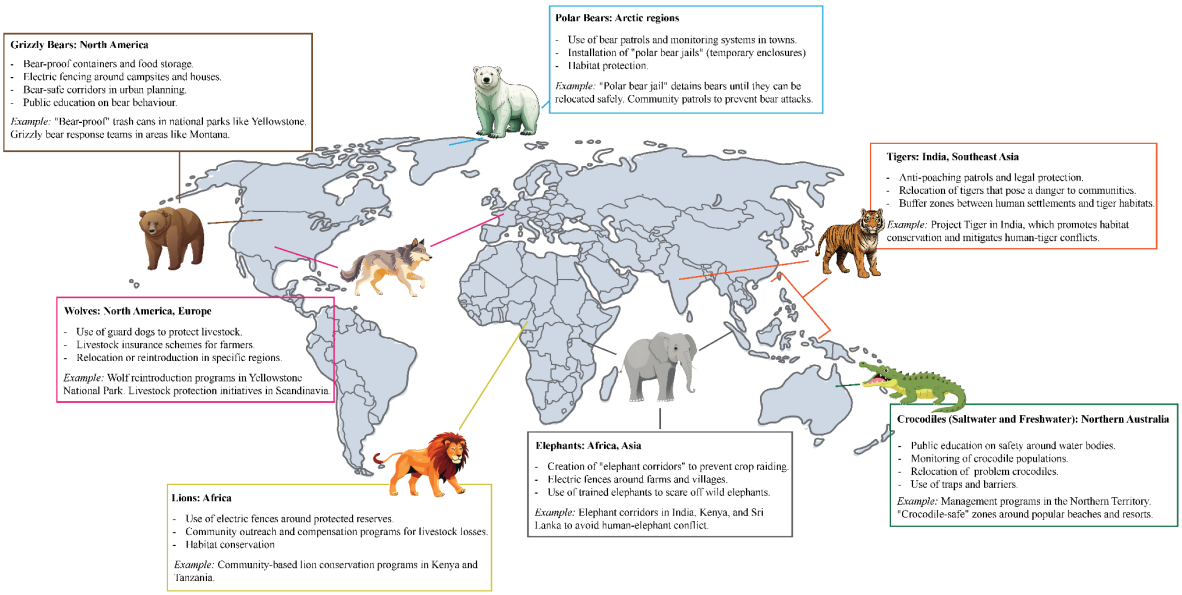

Figure 2: Examples of 'problematic' species around the world and the 'compromise' solutions that are used for management.

The early success that community role-players had with shark spotting publicly demonstrated an effective, socially, and ecologically acceptable non-lethal solution to managing shark risk (Pepin-Neff, 2019). This elevated the public perception of non-lethal management as a viable option, despite the availability and some public pressure for the implementation of traditional lethal shark control methods (Oelofse & Kamp, 2006). Ultimately, the implementation of this novel community-driven solution facilitated a compromise between continuing to "do nothing" and culling white sharks (Figure 1), thereby balancing recreational water user safety with white shark conservation principles. Through subsequent authority-led expert scientific workshops and stakeholder engagements, Shark Spotters was formalised and adopted as Cape Town's primary shark safety strategy in 2006. With local municipal funding, operations were scaled to cover high-risk beaches, creating sustainable employment for > 50 people from under-resourced communities (Kock et al., 2012). Shark Spotters also adopted a holistic approach to managing shark-human

conflict. This included applied research to better understand white shark ecology and the spatiotemporal risk of shark-human overlap (Hewitt et al., 2018; Kock et al., 2013; Weltz et al., 2013), as well as a comprehensive community education program and social media outreach where water users have been empowered to make informed decisions around shark risk and take individual responsibility for their safety when using the ocean.

Harnessing this approach has facilitated adaptive management and mitigation of dynamic shark risk in South Africa based on spatial and temporal fluctuations in white shark activity. This includes operating at certain beaches seasonally in response to water user density, as well as the expansion of Shark Spotters to Plettenberg Bay, an emergent area of risk associated with shifts in white shark distribution (Bowlby et al., 2023), following two fatal shark bites in 2022. Furthermore, in response to evolving community perceptions of risk, a non-lethal shark exclusion barrier was designed and seasonally deployed at Fish Hoek beach, Cape Town, in 2013 (Kock and O'Riain, 2015). This management intervention followed a non-fatal shark incident involving a swimmer who disregarded safety advice and beach closures despite the presence of sharks being recorded by spotters. This emphasises the need for flexible compromise solutions. While shark spotting is not applicable to all areas, due to the need for significant elevation close to shore and other factors influencing spotting efficacy (Engelbrecht et al., 2017; McPhee et al., 2021), the holistic principles of the program (i.e., the combination of safety, research, education, communities and conservation) are fundamental to effectively implement and maintain any compromise strategy for the sustainable management of water user safety and white shark conservation.

These principles have also been drawn upon to implement shark bite mitigation that are consistent with the 'compromise' policy on the east coast of Australia (Figure 3).

Following a spate of shark bites across relatively short temporal periods in 2015 off New South Wales, a government-commissioned independent review of emerging technologies for the protection of water users was undertaken (McPhee & Blount, 2015). The review was then considered at a shark summit, where >70 shark experts from Australia, South Africa and Hawaii identified the emerging bather protection technologies that should be considered for trialling at beaches in New South Wales, with the aims of transitioning away from a 'cull only' strategy (Figure 1). These efforts led to the development, testing and implementation of a shark bite mitigation strategy using non-lethal methods, with the objective of increasing protection of ocean users in New South Wales beaches, while minimising harm to sharks and other marine animals (Martin et al., 2022; Tate et al., 2021). The strategy, referred to as the "New South Wales Shark Management Strategy 2015-2020" (sharksmart.nsw.gov.au), employed the use of aerial surveillance including drones (Colefax et al., 2020; Butcher et al., 2019; Kelaher et al., 2020), Shark-Management-Alert-in-Real-Time (SMART drumlines; (Tate et al., 2021; Butcher et al., 2023), and real-time detection alerts of the presence of acoustically-tagged sharks via satellite-linked receiver stations (Spaet et al., 2020; Smoothey et al., 2023) as some of the compromise tools to improve bather protection and ecological impacts (Figure 3). The Shark Management Strategy was also subject to an independent review (Cardno, 2022), which helped inform the development and implementation of the 2022–26 Shark Management Program, which includes 305 SMART drumlines in 19 local government areas, drone patrols by Surf Life Saving groups at up to 50 beaches during NSW school holidays, 37 listening stations for tagged sharks, and partnerships with Surfing NSW to provide trauma kits, drones and associated training. The 2022–26 program also aimed to increase community awareness and education through a state-wide SharkSmart community

education awareness campaign and the SharkSmart app, as well as facilitating shark research and social studies (https://www.sharksmart.nsw.gov.au/current-program) (Figure 3). Since commencement of trials under the NSW Shark Management Strategy in 2015, some interactions with sharks have occurred at beaches with tagged shark listening stations, SMART drumlines and at beaches with traditional nets; however, there have been no bites while drones were deployed (Huveneers et al., 2024). A serious interaction between a white shark and a surfer at Port Macquarie, NSW, in August 2023 was the first incident with a target shark (i.e. white, tiger or bull shark) while the SMART drumlines were deployed. This was followed by another white shark bite in July 2024 at North Wall, Port Macquarie. The NSW Shark Management Strategy indicates that broad stakeholder engagement and scientifically supported mitigation efforts can contribute to broad-scale shark bite mitigation efforts to increase bather protection while minimising harm to sharks and other marine species. However, reviews of the strategy (Cardno, 2022; Huveneers et al., 2024) found that area-based protection alone may not be sufficient for eliminating interactions with white sharks and other target sharks. Instead, a hierarchical approach emphasising personal responsibility when entering the ocean, underpinned by evidence-based decision making, can further reduce risk for beachgoers, including matching mitigation strategies for specific high-risk water-users (i.e. kayakers, surfers, swimmers, or professional divers). For example, telemetry data may show seasonal trends in the occurrence of white sharks in coastal areas and can contribute to proactive management for beachgoers in areas and times of relative increases in shark abundance (Bastien et al., 2020, Gooden et al., 2025b; Lee et al., 2021; Winton et al., 2021). Additionally, research on effectiveness of personal electric deterrents has led to a shark deterrent rebate for residents in Western Australia to receive a $200 rebate

when they purchase scientifically-tested deterrent products (sharksmart.com.au/staying-safe/rebate-faqs). However, there is currently little evidence connecting the implementation of rebates and use of personal shark deterrents in minimising shark bites at this early stage.

Figure 3. The range of tools trialled since 2015 in the New South Wales, Australia shark management program.

These examples demonstrate the progress made to achieve compromise white shark management and policy solutions, and while strategies may differ regionally, there is an overarching need to understand stakeholder beliefs and values. The recently established framework by Henriksen et al., (2025), which assesses stakeholder-specific views on shark bite risk and mitigation strategies, presents an ideal approach for policy makers to consider and engage with as part of the process. For a white shark management strategy to be viable, policies must be representative of all stakeholders and agencies, be regionally and culturally relevant, have minimal socio-economic impacts, and remain sensitive to both the human risk perspective and the conservation status and ecological role of the white shark. Relevant stakeholder groups to engage with may encompass community organisations (such as surf lifesaving, surfing and recreational fishing groups, conservation organisations), businesses (including tourism and ecotourism, commercial fishing and aquaculture), Indigenous communities, government bodies (councils, police, state agencies), as well as researchers and scientists. While policy makers may face challenges with engaging the broader public, policies that are developed transparently, account for diverse perspectives, and are evidence based are more likely to achieve a consensus among stakeholders to reach a compromise.

Thus, achieving a balance between culling and conservation in white shark management necessitates a holistic approach which includes education and outreach to facilitate discussions and foster fact-based dialogue among all stakeholders. However, the dissemination of information is also important to ensure that accurate and useful information

is provided and accessible to the relevant audience. Policy makers should also aim to determine the degree to which water-users use that information to make informed decisions about where and when to swim, surf and dive. When used appropriately, media outlets can be a powerful tool for reaching a wide audience to relay relevant information; however, media framing of shark bites can be negative, sensationalised and increase public anxiety and negative perception of sharks (Cermak, 2021; Lucrezi et al., 2019; Philpott, 2002; Sabatier & Huveneers, 2018). This highlights the need for conservation scientists and government agencies to better engage with media so that information and advice presented via media outlets is accurate and effective, rather than being sensationalised and fear-mongering (Nel & Peschak, 2006; Muter et al., 2013). Information should also be accessible and digestible to the general public as some sources may not reach or be interpretable to most ocean-users (e.g., scientific articles). Increasingly, social media outlets (e.g., Sharks4kids; Atlantic White Shark Conservancy; instagram.com/a_whiteshark/?hl=en; NSW SharkSmart; instagram.com/nsw_sharksmart; OCEARCH; https://www.instagram.com/ocearch/?hl=en), mobile phone applications (e.g., NSW SharkSmart app; sharksmart.nsw.gov.au; Sharktivity; atlanticwhiteshark.org; OCEARCH Global Shark Tracker; https://www.ocearch.org/tracker/), or community information sessions are effective means of communication for the general public. Greater shark awareness and information are needed to reduce personal risk for beachgoers, whether it be to avoid swimming at times of the day with higher risk, when to consider investing in a personal deterrent, or how to provide suitable and timely first aid in the event of a shark bite (Huveneers et al., 2024). Cultural differences also need to be accounted for in education; members of the public who inhabit urban areas and rarely frequent ocean areas are likely to

differ in opinion than those who live in smaller coastal communities, so conveying education messages should account for these differences and be tailored to the target audience.

Another key component to education in white shark management is the willingness to be open to feedback from relevant stakeholders. Arranging education spaces and outreach programs where dialogues are two-way functioning will provide useful feedback for informing shark management and associated policy. Acceptance of management strategies and recommendations will improve when representative bodies can have an influence in decision-making processes and all relevant stakeholders are represented. Public sentiment largely holds the government responsible for the protection of ocean users from shark bites (Crossley et al., 2014; Martin et al., 2022), so governments should provide people with opportunities to make informed decisions, while recognising and balancing the responsibility for personal decisions about entering the water, particularly in remote locations (Huveneers et al., 2024; Martin et al., 2022). Effective education on shark bite risk and management, predominantly led by effective communication, therefore offers an opportunity to complement efforts from research outcomes, government policies, and personal responsibility to further mitigate risk when entering the water, while maintaining conservation objectives.

Alternative and adaptive management approaches used on other animals

Lethal approaches have been long used against species responsible for real or perceived threats to human interests. Predator abatements for terrestrial carnivores were common to reduce the loss of livestock, human death or injury, or damage to infrastructure and crops from animals such as wildcats, elephants, wolves, crocodiles and bears (Pooley et al., 2017). Such an anthropocentric perspective to tackle human-wildlife conflicts started to

change during the 1990s and 2000s, when research and management strategies began to focus more on co-existence (Robinson, 2005; Woodroffe et al., 2005). As shown in Figure 3, alternatives to lethal controls comprise solutions that are efficient and have minimal lateral environmental consequences, and that are selective towards the problematic individuals/species (McManus et al., 2015). Management of human-wildlife conflicts depends on understanding the complex relationships between the ecological and social aspects involved in the different scenarios (Carter at al., 2014). Strategies should also account for human experiences and perceptions (i.e., beliefs and emotions) towards the species involved, where in some instances, positive experiences may buffer the negative effects of interactions and help achieve co-existence (Teixeira et al., 2021).

Management of ecological resources is more likely to be successful under adaptive management approaches that respond to community concerns rather than fixed approaches due to the uncertainties involved, such as environmental and sampling variations, and the different responses of individuals/populations to the strategies enacted (Franklin, 2007). Adaptive management approaches to manage human-wildlife conflicts are more frequent in North America (43%), followed by Europe (25%) and Australasia (22%), with mammals being the most common animal group involved (45%), followed by birds (23%) and fish (18%) (Richardson et al., 2020). In addition, adaptive management based on empirical evidence and using non-lethal approaches (e.g., active guarding, fencing, repellents) can lead to benefits to both humans and animals, thus helping achieve co-existence outcomes (Killion et al., 2021).

Future directions and recommendations

This review highlights the complexity of managing human-shark conflicts and reaching a resolution that supports the sustainable co-existence of humans and white sharks in the marine environment. Management decisions relating to white shark populations and human-shark interactions can be highly controversial given the ethical, ecological, cultural and practical considerations and often polarised views of different stakeholder groups (Stokes et al., 2020; Atkins et al., 2023). By definition, the white shark conundrum is a 'wicked problem' (i.e., a complex problem with conflicting aims and no clear resolution without conflicting consequences; Niella et al., 2021). This synthesis paper showed that the traditional policy positions of conservation or culling will not resolve the white shark conundrum alone. Instead, evidence based on five decades of experience suggests that a 'compromise' policy involving the adoption of non-lethal and both adaptive and tailored shark mitigation measures is most appropriate in the 21st century.

In essence, there is no silver bullet solution for every region and circumstance given the complexity and multi-faceted nature of managing human-shark conflicts. For example, drones (e.g. Butcher et al., 2019) or continuous visual surveillance (e.g. Engelbrecht et al., 2017) may be suitable measures for reducing shark-human interactions at populous swimming/surfing beaches, whereas SMART drumlines might be useful at highly frequented beaches with easy water access for response operators. Yet, neither is appropriate for remote coastlines sporadically used by recreational water users and commercial operators, where personal deterrents and/or improved first response measures may be more suitable (Huveneers et al., 2018; McPhee et al., 2021). Further, while some mitigation measures have shown effectiveness in reducing shark bite risk (Chapuis et al., 2019; Engelbrecht et al., 2017; Marcotte & Lowe, 2008; O'Connell et al., 2014; Ryan et al., 2024;

Thiele et al., 2020), no single strategy gives complete protection. For this reason, a combination of different measures should be implemented that are best suited to a specific region and community (McPhee et al., 2021). These measures should be selected based on peer-reviewed, credible scientific and published research, stakeholder opinions and desired public outcomes to ensure an adequate reduction of bite risk.

We recommend policy makers use a decision support tool that considers human safety, social, economic, and environmental impacts in selecting the most appropriate shark-bite mitigation measures for a selected area (e.g., Henriksen et al., 2025). This includes educating the general public about white shark biology, ecology and behaviour, which will help to reduce fear and to explain how shark mitigation measures work (and what they cannot achieve), and what people can do to reduce their own risk (e.g., avoid entering the water in certain conditions/times/places or invest in a scientifically-tested personal deterrent device). Also, communications should be framed around reducing risk instead of creating unrealistic expectations that entering the ocean can be risk-free. We also recommend the establishment of shark-bite mitigation working groups that encompass representatives of local stakeholders (e.g., including government, scientific, recreational, cultural groups, and industry members) in regions where shark-bite mitigation is implemented or proposed. This would include a framework for monitoring and measuring the effectiveness of shark-bite mitigation measures that are trialled or implemented, noting that measures of effectiveness are usually based around measuring the effect on intermediate outcomes, such as the ability to reduce spatial overlap between sharks and humans, or the ability to affect shark behaviour, and not the ultimate outcome which is reducing the number of shark bites.

However, analysing shark bites with statistical certainty is challenging due to the rare nature of incidents (Huveneers et al., 2024).

We emphasise the importance of transparent and inclusive decision and policy making processes that consider the views and values of all relevant stakeholders. Such approaches are likely to provide the best chance of reaching a successful compromise between stakeholders and meeting management objectives that promote the co-existence of sharks and humans in the marine environment. We also acknowledge that public perception has and continues to have a significant influence on shark management responses. However, public perception is often influenced by media channels that use sensationalised and fear-mongering messaging, which can lead to risks of shark bites being misunderstood, and adverse outcomes for white sharks and other non-target species. This highlights the importance of effective engagement between scientists, government agencies, and the media so that information and advice presented via media channels is accurate and avoids disproportionate responses from the public.

There is an increasing urgency to reach compromise management strategies that promote white shark and human co-existence in different regions of the world, particularly those where white shark populations have suffered significant declines and recovery trajectories remain uncertain and/or where human-shark interactions continue to impact local communities. This is particularly pertinent considering the functional importance of white sharks in the regulation of marine biodiversity and ecosystem function, and the potential cascading ecosystem consequences of white shark declines.

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