Epidermal Microbiomes of *Triakis Semifasciata* Are Consistent Across Captive and Wild Environments

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Abstract

Background: Characterizations of sharks-microbe systems in wild environments have outlined patterns of species-specific microbiomes; however, whether captivity affects these trends has yet to be determined. We used high-throughput shotgun sequencing to assess the epidermal microbiome belonging to leopard sharks (*Triakis semifasciata*) in captive (Birch Aquarium, La Jolla California), semi-captive (<1 year in captivity; Scripps Institute of Oceanography, California) and wild environments (Moss Landing and La Jolla, California).

Results: Here we report captive environments do not drive microbiome composition of *T. semifasciata* to significantly diverge from wild counterparts as life-long captive sharks maintain a species-specific epidermal microbiome resembling those associated with semi-captive and wild populations. Major taxonomic composition shifts observed were inverse changes of top taxonomic contributors across captive duration, specifically an increase of *Pseudoalteromonadaceae* and consequent decrease of *Pseudomonadaceae* relative abundance as *T. semifasciata* increased duration in captive conditions. Moreover, we show captivity did not lead to significant losses in microbial α -diversity of shark epidermal communities. Finally, we present a novel association between *T. semifasciata* and the *Muricauda* genus as MAGs revealed a consistent relationship across captive, semi-captive, and wild populations.

Conclusions: Our report illustrates the importance of conservation programs for coastal fishes as epidermally-associated microbes of near-shore shark species do not suffer detrimental impacts from long or short-term captivity. Our findings also expand on current understanding of shark epidermal microbiomes, explore the effects of ecologically different scenarios on benthic shark microbe associations, and highlight novel microbial associations that are consistent across captive gradients.

Introduction

The eukaryotic organism harbors an astounding number of microbes both internally and externally to supplement nutrient breakdown, thwart pathogenic colonization, and provide defense via molecular byproducts, and is known as the microbiome. The eukaryotic host and the respective microbiome has been reclassified as a meta-organism, representing a functional system that adapts to changing environments and external threats by cultivating a synergistic interdependence with microscopic symbionts (Bosch and McFall-Ngai 2011). The epidermal microbiome of the marine metaorganism reflects both environmental conditions and host-microbiome interdependent effects, in addition to directly interfacing with seawater, while remaining distinct from the reservoir of microbes in the water column. The epidermal microbiomes belonging to triple fin fish (*Forsterygion capito*) experiencing polluted conditions, for example, have lower levels of biodiversity compared to fish residing in pristine environments (Montenegro et al. 2020). Likewise, microbial profiling of stranded leopard sharks (*Triakis semifasciata*) revealed infections caused by the pathogenic bacteria *Miamiensis avidus*, thus causing the mass die-offs of *T. semifasciata* populations in the San Francisco Bay the summer of 2017 (Retallack et al. 2019). While the presence of unicellular species is telling, metabolic potentials of epidermal

microbiomes is equally descriptive of environmental conditions. The epidermal microbiomes belonging to thresher sharks (*Alopias vulpinus*) encoded higher levels of genes associated with heavy metal resistance (Doane et al. 2017, 2020), indicating an increased supply of cobalt, zinc, cadmium, and iron from the host as Chondrichthyan (cartilaginous) fishes are known to attain heavy metals in the integumentary tissues (Jeffree et al. 2006). Therefore, the taxonomy and functional profiles of epidermal microbiomes to serve as indicators of environmental conditions, disease, and the provision of host-supplied nutrients. To this point, data for microbiomes belonging to captive, vertebrate hosts is still lacking, and is exceptionally dearth for the Chondrichthyan clade.

Extant sharks (Chondrichthyes subclass: Elasmobranchii, superorder: Selachimorpha) are vertebrates showcasing an ancient host-associated microbiome evolution in addition to harboring diverse biophysical and physiological traits such as the ability to naturally produce bioluminescence via photophores (Gruber et al. 2016) and the ability to detect the electromagnetic fields produced by all living organisms using ampullae of Lorenzini (Kalmijn 2019). The shark epidermis, in particular, is exceptional due to the presence of overlapping dermal placoid scales (denticles) connected to numerous, horizontally-arranged layers of muscles that are known to contract, thereby optimizing the build-up of hydrostatic pressure during fast swimming (Bechert, Bruse, and Hage 2000; Meyer and Seegers 2012). The physiology of the shark epidermis supports protective functions similar to those of Actinopterygii (Ray-fin fish), whereby continuous shedding of denticles, in concert with low drag and the flexion of the scales, reinforces antifouling efforts. The shark epidermis is not completely free of biofilms; although elasmobranchs produce a reduced mucosal layer relative to teleost fish, several species including *T. semifasciata*, *A. vulpinus*, the whale shark (*Rhincodon typus*), and the round ray (*Urobatis halleri*) have been observed to have a microbiome that mirrors the respective evolutionary trajectory of the host (Doane et al. 2020). This concept by which the microbiomes reflect host phylogeny is called phylosymbiosis. The combination of the physical and chemical barrier that is the mucosal layer, acting jointly with a maintained microbiome across evolutionary time, may account for the few reported cases of infections witnessed in wounded, wild black-tip reef shark (*Carcharhinus melanopterus*) populations (Pogoreutz et al. 2019), where pathogens were absent in the epidermal microbiome. Investigations into shark-associated epidermal microbiomes, therefore, are necessary to supplement the scarcity of information concerning the shark metaorganism.

While host-related factors including biological sex, health status (Egan et al. 2018), and diet. (Antwis et al. 2014) regulate microbial recruitment and retainment, environmental such as geography, pH, and salinity also contribute to the composition of microbial communities and functions (Haggerty and Dinsdale 2017; Ross, Rodrigues Hoffmann, and Neufeld 2019). Investigating microbiomes of sharks in captivity environments such as public aquariums, provide consistent conditions with reduced introduction of natural microbial communities due to water filtration, stable macroscopic species interactions, regular diets, and lower variations of abiotic factors that influence the composition of both host-associated, and water column, microbiomes. Of the 1370 species of Elasmobranchs, only a small percentage of associated microbial communities have been characterized including those previously mentioned in addition to the nurse (*Ginglymostoma cirratum*), lemon (*Negaprion brevirostris*), sandbar (*Carcharhinus*

pleumbeus), caribbean reef (*Carcharhinus perezii*), and tiger (*Galeocerdo cuvier*) sharks and the thornback (*Raja clavate*) and round (*Urobatis halleri*) stingrays (Doane et al. 2017, 2020; Storo et al. 2021). A single study of a captive population was performed with cownose rays (*Rhinoptera bonasus*) exclusively residing in an aquarium touch tank (Kearns, Bowen, and Tlusty 2017). Herein, we investigate the impact that a captive environment exerts on the epidermal microbiota associated with captive *T. semifasciata* individuals and compare the composition and functional potentials of microbiomes belonging to wild sharks that live in nearshore habitats.

The *T. semifasciata* species was chosen as our model to investigate the change in microbiome composition across environments as they commonly inhabit both aquaria year-round and aggregate along the coast of southern California in the spring, summer, and fall months (Nosal et al. 2013). The *T. semifasciata* elasmobranch species has also been shown to have a phyllosymbiotic relationship with their epidermal microbiomes (Doane et al. 2020). However, describing the epidermal microbiomes of *T. semifasciata* across captive and wild environmental gradients has yet to be accomplished. Therefore, the proximity of *T. semifasciata* populations along coastal waters and those concomitantly housed in nearby research facilities provided us a unique opportunity to sample epidermal microbiomes of captive (>1 year in captivity), temporarily captive (<1 year in captivity), and wild individuals using shotgun metagenomics. Our analyses show the taxonomic composition of the epidermal microbiomes belonging to *T. semifasciata* remain consistent between captive sharks and wild peers and irrespective of captive conditions. Furthermore, we show broad functional genes remain largely unchanged across environments and host lifestyles, as associated bacteria perform comparable functions, differing in few specific functions only which may reflect environmental conditions and resources and host-derived nutrients. Last, by constructing metagenomes assembled genomes, we confirm the presence and novel association of specialist microbes that are consistent across wild and captive sharks.

Methods

Sampling of Metagenomes

Wild *T. semifasciata* were sampled in the summers of 2013 ($n = 6$) and 2017 ($n = 8$) at La Jolla Shores in San Diego, California (32.868342, -117.255304), and the summer of 2015 ($n = 5$) at Moss Landing in Monterey County, California (36.801588, -121.791970). Wild sharks were sampled during the summer aggregations that are characteristic of *T. semifasciata* populations (Nosal et al. 2013, 2014). Wild sharks were initially caught using a handline with baited barbless circle hook and, using a scoop net, brought onboard for sampling and subsequent release. Four *T. semifasciata* ($n = 4$) were sampled in short term captivity (< 1 year) during the summer of 2016 while cohoused in a tank at the Scripps Research Institute of Oceanography in San Diego, California (32.865402, -117.252945). Captive *T. semifasciata* ($n = 4$) were sampled in the summer of 2018 at the Birch Aquarium at Scripps Institution of Oceanography in La Jolla, California (32.865658, -117.250535) to represent microbiomes belonging to *T. semifasciata* long held (>1 year) in captivity. Captive sharks were corralled by divers into a pen and immobilized in a sling during sampling.

To consistently describe the shark epidermis-associated microbiome, microbes were collected between the pectoral fin and dorsal fin along the lateral line on the left side of the organism (Fig. 1.A) using a blunt, closed-circuit syringe. The syringe is prefilled with the 100 kDa filtered seawater that was then flushed over the epidermis to dislodge microbes without introducing microbes from the environment (Figure 1.B; Doane et al. 2017). The approximate 200 mL of captured microbe was collected on a sterivex, where one sterivex per individual was obtained each collection date.

To describe the water-associated microbial communities, bulk water samples were taken: ~60 L of tank or ocean water were simultaneously collected and filtered once through a nylon mesh sieve (200 µm pore size) to remove large debris and eukaryotic organisms and again using tangential flow filtration (TFF, 100 kDa) to further concentrate the microbial consortia (Dinsdale et al. 2008). The resulting sample of approximately 500 mL composed the concentrated sample and was processed and collected on a Sterivex filter (0.22 µm).

DNA Extraction and Metagenome Sequencing and Annotation

Upon collection, cells were lysed within the sterivex filters and purified using a Macherey Nagel Tissue Kit. Microbiome DNA was then prepared for shotgun metagenomic sequencing using the Swift 2S Plus Kit (Swift Biosciences). Purified DNA was sequenced using an Illumina MiSeq sequencer with Mi-Seq v3 Reagent Kit on site at San Diego State University as previously performed (Dinsdale et al. 2013; Doane et al. 2017; Haggerty and Dinsdale 2017). Following sequencing, reads were processed for quality control via PrinSeq to remove artificial duplicates, and reads having >10 N's and <60 bp as per previous metagenomic workflows (Schmieder and Edwards 2011). High quality, paired reads were uploaded to Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) to call taxonomic and functional gene assignments. MG-RAST accomplishes gene calling and protein prediction with BLAST comparisons to the NCBI and SEED protein databases (Overbeek et al. 2014). Sequencing annotations were conducted using the following parameters: e-value $>10^{-5}$, 70 percent identity, and > 60 bp alignment length.

MAG Assembly and Annotation

Metagenome assembly genomes were constructed to identify whether metagenomes from the sharks across captivity and wild environments contributed sequences to the genomes, thus potentially identifying shark specific microbes. The MAGs were generated from metagenomes belonging to 38 individual *T. semifasciata* across captivity status using MEGAHIT (Li et al. 2015). Contigs >1000 bp were run through METABAT2 binning program to reconstruct MAGs. CheckM was applied to each MAG and for the remainder of this article, only MAGs with at least 80 % completeness and less than 10 % contamination (confirmed via CheckM; Papudeshi et al. 2017; Parks et al. 2015, 2017) will be presented and discussed following quality control metrics outlined in previous investigations (Nayfach et al. 2020; Robbins et al. 2019). Bacterial delineations of MAGs were compared across several taxonomic classification algorithms including FOCUS (Silva et al. 2014), CheckM, and the Pathosystems Resource

Integration Center (PATRIC; Wattam et al. 2014), and confirmed by comparing average nucleotide identity (ANI; Yoon et al. 2017) or DNA Kmer-based evidence of similarity surpassing 90 %, reflective of standard criteria (Chun and Rainey 2014). Of the 54 bins, eight met baseline requirements for further analysis, with three MAGs taxonomically classified with high confidence (> 90 %) and three identified with low (< 90 %) confidence. We utilized the advanced analysis and visualization tool *Anvi'o* to visually explore and interpret our assembly-based metagenomes (Eren et al. 2015). Functional gene annotation of MAGs was performed through PATRIC using the BLAT alignment tool.

Statistical Analyses

All taxonomic data was normalized to produce relative abundance data and was then fourth root transformed to reduce variation among samples. The α -diversity indices of microbial community richness, evenness, and diversity were measured using Margalef's (d), Pielou's (J'), and Inverse Simpson's ($1/\lambda$) indices, respectively (Chao and Shen 2003; Death 2008; Pielou 1966) to discern the effect captivity exerts over associated microbial community biodiversity. To measure the intraspecific similarities, and between group dissimilarities, similarity percentages breakdowns (SIMPER) were calculated (Clarke 1993). To measure variation community compositions between environmental groups a permutation multivariate analysis of variance (PERMANOVA) using 999 random permutation tests was utilized (Kelly et al. 2015). Association mapping between metagenomes across environments were visualized via non-metric multidimensional scaling (nMDS) generation with correlation overlays (Kruskal 1964). Tests for differences in group dispersion or homogeneity of the multivariate variations were generated using a PERMDISP analysis (Anderson 2006; Clarke and Gorley 2015).

Results

From the epidermal microbiomes of 27 *T. semifasciata* individuals, 31 114 584 sequenced reads were identified as bacteria and archaea spanning 27 phyla, 42 classes, 92 orders, 208 families, and 564 genera (Table 1). The total number of families present for *T. semifasciata* metagenomes ranged from 164 to 208 phyla. Water-associated microbiomes significantly differed from host-associated microbiomes in taxonomic composition (PERMANOVA: Family, Pseudo- $F_{df = 1, 31} = 2.056$, $P(\text{perm}) < 0.05$). The water column microbial communities had little within-group variation (SIMPER analysis; 72.96 similarity) and high dissimilarity and are not compared further.

Table 1
Base pair and sequence count for *T. semifasciata* across environments.

Sample	Base Pair Count	Sequence Count
Captive 1	13,254,680	36,040
Captive 2	165,572,548	563,299
Captive 3	129,410,990	454,887
Captive 4	140,813,416	512,408
Semi-Captive 1	194,456,844	683,590
Semi-Captive 2	114,129,299	364,329
Semi-Captive 3	106,800,378	351,397
Semi-Captive 4	147,769,235	491,188
Wild 1	263,295,773	1,298,868
Wild 2	262,597,687	1,176,351
Wild 3	258,939,703	1,226,960
Wild 4	653,532,367	2,531,410
Wild 5	640,031,260	2,434,754
Wild 6	673,699,889	2,549,181
Wild 7	397,391,784	1,368,629
Wild 8	273,016,668	1,039,793
Wild 9	291,042,619	1,045,530
Wild 10	291,228,603	1,001,807
Wild 11	369,123,866	1,254,908
Wild 12	211,033,215	710,743
Wild 13	279,527,632	914,034
Wild 14	195,358,159	648,568
Wild 15	109,131,187	328,208
Wild 16	94,795,257	316,833
Wild 17	186,632,228	623,111
Wild 18	342,901,940	1,246,300

Sample	Base Pair Count	Sequence Count
Wild 19	217,511,438	709,664

Microbial community richness (d), evenness (J'), and overall diversity ($1/\lambda$) were similar across captivity status (Table 2). To understand the taxonomic composition and variation across environments we then characterized each group of metagenomes. The major contributors to taxonomic classes of epidermal microbiomes belonging to *T. semifasciata* long held in captivity were *Alteromonadales* (57.1% $\pm$ 4.29 S.E.), *Burkholderiales* (5.91% $\pm$ 0.79) and *Sphingomonadales* (5.51% $\pm$ 0.85), belonging to Gamma-, Beta-, and Alphaproteobacteria clades respectively. Of the marine generalists, the *Pseudoalteromonadaceae* family were the most abundant family present (30.1% $\pm$ 2.42), followed by *Alteromonadales* (20.01% $\pm$ 1.60), *Sphingomonadaceae* (4.48% $\pm$ 0.73), and *Shewanellaceae* (2.62% $\pm$ 0.14; Figure 2). The epidermal microbiomes of *T. semifasciata* inhabiting the southern coast of California harbored different abundances of major clades than their captive counterparts; fine-scale taxonomic resolution revealed differences between captive and wild benthic shark microbiomes were accounted for by major taxonomic contributors (>1% relative abundance), with smaller populations having no significant impact on microbiome compositions. While the *Pseudoalteromonadaceae* family dominates the skin microbiomes of *T. semifasciata* residing in captivity (21.4 $\pm$ 1.45 S.E.), the *Flavobacteriaceae* family contributed the most to metagenome compositions belonging to wild *T. semifasciata* (10.13 % $\pm$ 1.98), followed by *Pseudomonadaceae* (8.86 % $\pm$ 1.87) and *Alteromonadaceae* (7.26 % $\pm$ 0.94). The *Pseudomonadaceae* (18.75 % $\pm$ 2.87) and *Flavobacteriaceae* (8.38 % $\pm$ 0.337) families were among the top three major taxonomic contributors to *T. semifasciata* microbiomes for semi-captive shark microbiomes, with the *Moraxellaceae* (11.26 % $\pm$ 2.12) family comprising significantly more of the microbial composition than in other groups.

Table 2

Table 1 Average biodiversity indices for epidermal microbiomes belonging to *T. semifasciata* across environments.

Host Environment	Margalef's (d) Index $\pm$ S.E.M.	Pielou's (J) Index $\pm$ S.E.M.	Inverse Simpson ($1/\lambda$) Index $\pm$ S.E.M.
Captive	41.21 $\pm$ 4.15	0.59 $\pm$ 4.3E-02	70.50 $\pm$ 3.54
Semi-captive	41.53 $\pm$ 3.54	0.624 $\pm$ 3.37E-02	73.30 $\pm$ 2.27
Wild	40.07 $\pm$ 1.44	0.581 $\pm$ 2.88E-02	68.29 $\pm$ 2.29

While no statistical differences were observed between wild and neither captive nor semi-captive epidermal microbiomes (PERMANOVA Family level, Pseudo-F_{df=2,18} =, P(permanova) > 0.1); Genus level, Pseudo-F_{df=2,18} =, P(permanova) > 0.1), SIMPER analyses revealed trends along captive durations: microbiomes belonging to captive sharks are more similar to those associated with semi-captive sharks (87.99 Average Similarity (A.S.) = 100 - Average Dissimilarity) than to wild sharks (84.27 A.S.), and semi-captive sharks are more similar to captive sharks than to wild sharks (85.6 A.S.). Dissimilarity between

captive and wild *T. semifasciata* was attributed to higher abundances of *Pseudoalteromonadaceae* in captive microbiomes, consistently driving differences between the two groups (3.04 Diss/S.D.). Taxonomic clades accounting for consistent differences between semi-captive and wild sharks were *Alcanivoraceae* (2.79 Diss/S.D.), *Planctomycetaceae* (2.47 Diss/S.D.), and *Moraxallaceae* (2.38 Diss/S.D.), and were higher in proportional abundance in semi-captive metagenomes. The constant contributor of dissimilarity between captive and semi-captive individuals was attributed to *Halomonadaceae* (3.74 Diss/S.D.), which was approximately twice the relative abundance in semi-captive epidermal microbiomes. There was no difference in the microbiome variation of *T. semifasciata* epidermal microbiomes residing in each environment (PERMDISP: Pseudo-Family, $F_{df=2,18} = 1.508$, $P(\text{perm}) > 0.1$, Genus, Pseudo-F $F_{df=2,18} = 1.32$, $P(\text{perm}) > 0.1$). The microbial families of *T. semifasciata* epidermal microbiomes did not form distinct clusters for each environment when visualized using an nMDS; the structure of *T. semifasciata* epidermal microbiome data across captivity showed no difference in coefficients of similarity and several microbial families were found to be highly correlated (> 0.85 ; Figure 3). These families include *Pseudomonadaceae*, *Shewanellaceae*, and *Sphingomonadaceae*, and represent major taxonomic contributors ($>1\%$) for all three groups of *T. semifasciata*. Less represented clades ($<1\%$) related to the spread of metagenomes include *Beijerinckiaceae*, *Brucellaceae*, *Rickettsiaceae*, and *Bacteroidaceae* as illustrated in nMDS overlays (Figure 3).

Comparisons of functional gene potentials of *T. semifasciata* epidermal microbiomes across environments

There were between 661 and 778 metabolic categories identified in all *T. semifasciata* metagenomes. Water column metagenomes functional potentials were significantly distinct from *T. semifasciata* epidermal microbiomes (PERMANOVA: subsystem level 1, Pseudo-F $F_{df=1,31} = 1.93$, $P(\text{perm}) < 0.05$) and were not compared further.

Of the 27 major gene pathways identified in *T. semifasciata* metagenomes, six contributed $>5\%$ of reads, with the two top contributors ($>10\%$) involving carbohydrate ($12.2\% \pm 0.196$ S.E.) and amino acid ($11.0\% \pm 0.151$) metabolism. Analyses of functional levels revealed no statistical differences between all groups (PERMANOVA: subsystems level 3, Pseudo-F $F_{df=2,18} = 0.859$, $P(\text{perm}) > 0.5$). In addition, differences in similarity between groups were insignificant, as wild metagenome metabolic profiles were comparable (SIMPER: wild vs semi-captive, 90.02 A.S.; captive vs semi-captive, 91.42 A.S.) with the greatest dissimilarity measured between wild and semi-captive shark populations (90.4 A.S.) when analyzing metabolic pathways (SEED subsystem level 2). The functions contributing consistently to differences between wild and semi-captive epidermal microbiomes, as identified by SIMPER analysis, were genes involved in gene transfer agents (1.95 % contribution, 1.95 Diss/S.D.), motility and non-flagellar swimming (1.69 %, 1.38 Diss/S.D.), and protein secretion system type VII (1.47 %, 1.35 Diss/S.D.).

Although β -diversity analyses revealed no statistical differences between functional profiles of metagenomes belonging to *T. semifasciata* epidermal microbiomes across environments, several

functional pathways were found to differ. Captive shark metagenomes featured increases in relative abundances of genes; in carbohydrate synthesis ($14.06\% \pm 0.24$ S.E.) and utilization, such as the more specific fermentation ($0.959\% \pm 0.06$; Figure 4) pathway (SEED subsystem: level 2). Specific functional pathways (SEED subsystem: level 3) featuring utilization of simple sugars and sugar alcohols were also significantly increased in captive metagenomes including fructose and mannose inducible phosphotransferase system (PTS; $0.458\% \pm 0.01$), methylglyoxal metabolism (0.37 ± 0.01), and mannitol metabolism ($0.192\% \pm 0.26$, Figure 5). Genes coding for enzymes involved in the breakdown of these saccharides i.e., beta-glucosidase ($0.48\% \pm 0.01$), were correspondingly increased in captive shark epidermal metagenomes compared to semi-captive and wild counterparts, in addition to the subsequent alcohol synthesis including acetone, ethanol, and butanol ($0.20\% \pm 0.09$). Pathways involved in heavy metal acquisition were likewise increased in captive shark microbiomes, as indicated by increased genes involved in heme/hemin uptake and utilization ($0.19\% \pm 0.03$), iron acquisition ($2.65\% \pm 0.17$). Furthermore, pathways involved in virulence, disease, and defense were significantly more abundant ($5.85\% \pm 0.03$), including specific genes related to periplasmic stress ($0.02\% \pm 0.01$), capsular polysaccharide biosynthesis and assembly ($0.13\% \pm 0.013$), murein hydrolytic activities ($0.341\% \pm 0.01$), and the antibiotic resistance gene BlaR1 family regulatory sensory-transducer disambiguation ($0.34\% \pm 0.32$). Conversely, higher functional potentials involved in vitamin synthesis ($7.22\% \pm 0.145$ S.D., $p < 0.01$) and nitrogen metabolism ($1.82\% \pm 0.001$) were observed in semi-captive samples. For *T. semifasciata* individuals, no significant gene pathways differed between semi-captive or wild metagenomes.

Metagenome Assembled Genomes constructed from Microbial Communities Associated with *T. semifasciata*

Cross assembly of the 27 *T. semifasciata* metagenomes yielded 54 MAGs containing 241 814 contigs greater than 1 kilobase pairs, with N50 of 735 bp and N75 of 583 bp. Of these, nine high quality MAGs were constructed spanning seven known bacterial phyla (Supplementary Table 1). While all *T. semifasciata* metagenomes were involved in MAG assembly, three groupings of contributions to MAG generation can be observed in Figure 6, where more even mean coverage of MAG contribution by *T. semifasciata* populations across environment is visualized. Among the groups, group 1 in Figure 6 highlights heavy contributions from both captive and wild metagenomes, while not harboring any of the nine high quality MAGs, while group 2 contains two MAGs further investigated. Finally, group 3 has an even spread of mean contribution from each metagenome environments and contains three high quality MAGs. The number of contigs wild shark hosts contributed to MAG assembly was greater ($87.1\% \pm 4.88$ S.E.) than both semi-captive ($7.45\% \pm 6.86$) and long-held captive sharks ($5.49\% \pm 4.50$) and is due to number of reads in the metagenomes.

Of the nine qualifying MAGs, two (Bin 27 and Bin 9) were identified as belonging to the *Muricauda* genus, an increasingly classified child taxon of *Flavobacteriaceae* family. Bin 27 had an average nucleotide identity match of 99.75% with the *Muricauda antarctica* species (Yoon et al. 2017), while identification of the species of Bin 9 remains incompletely verified (<90% similarity) at species level, with the highest resemblance matched to *Muricauda reustriensis* (84% similarity). The DNA G+C content of Bin 27 and

Bin 9 was 45.17% and 41.72% respectively, both falling within the acceptable range reported for taxa belonging to the *Muricauda* genus, i.e. 41-45.4 mol% (Arun et al. 2009). Bin 27 featured a 95.77% complete genome, with 4 106 genes, 4.23 % genome redundancy, and a total length of 4 285 655 bp. The Bin 9 MAG was calculated to have a 91.55 % complete genome composed of 4 673 genes, with more genomic redundancy (7.04%) and a longer total genome length of 4 625 638 bp (Figure 7). Following genome annotation, no resistance or susceptibility to antibiotics were found in Bin 27 encoding for *Muricauda antarctica*. However, two virulence features were discovered: GTP-binding and nucleic acid-binding protein YchF (fig|1055723.17.peg.1964), and ferric uptake regulation protein FUR (fig|1055723.17.peg.1945).

Of the remaining seven MAGs, two were of unknown taxonomic origin with no similar genomes (Bin 36 and 47). In decreasing order of confidence, the five remaining MAGs were identified as *Zunongwangia atlantica* (99.75%, Bin 30), *Roseivirga pacifica* (96.5%, Bin 30), *Leeuwenhoekiella blandensis* (63.9%, Bin 22), *Micavibrio spp.* (39.2%, Bin 13), and *Pseudomonas spp.* (34.4%, Bin 12; Supplementary Table 1). Finally, Bin 36 most closely resembled a member of the *Fluviicola* genus (23.34% similarity), while Bin 47 matched most (20% similarity) to the *Thalassospira* genus.

Discussion

Captive environments aim to conserve populations while balancing a complex ecosystem of micro- and macro-organisms. These efforts necessitate characterization of elasmobranch microbiomes across environments to understand the effect captive exerts on the meta-organism. We provide the first comparisons of taxonomic compositions and functional potentials between metagenomes associated with captive, semi-captive and wild *T. semifasciata* populations and show that captive environment exerts no detrimental pressure to *T. semifasciata* epidermal microbiome composition. In addition, there was no effect on biodiversity across environments. However, we did observe changes in microbial community structure as sharks increased captive status, and only more specific (SEED subsystem: level 2 and 3) functional pathways varied within metagenomes.

Epidermal Microbiome Taxonomic Structure as a Product of Captivity Duration and Host Factors

Here we show the composition of *T. semifasciata* populations differ in proportion of major contributors (>10% relative abundance) between captive and wild environments; we observed the *Pseudoalteromonadaceae* relative abundance to be significantly higher in captive samples, while their presence was only maintained in wild microbiomes. Consequently, we observed a shift in taxonomic compositions as *T. semifasciata* populations increased durations in captivity, including decreasing proportions of *Pseudomonadaceae* and *Moraxellaceae*, as *Flavobacteriaceae* and *Pseudoalteromonadaceae* became the dominant phyla in captive environments. The *Pseudomonadaceae* family, which belongs to the higher Gammaproteobacteria phylum and encompasses a diverse clade of microbes known to perform essential tasks such as nitrogen fixation and contaminant degradation. The

observed change from microbiomes dominated by generalists (*Pseudomonadaceae*) in wild environments to opportunists (*Pseudoalteromonadaceae*, *Flavobacteriaceae*) in captive metagenomes may signify wild environments drive the selection of fast-growing associates by providing an abundance of resources. Likewise, the increased relative abundance of the copiotrophic *Pseudomonadaceae* in wild groups may reflect unclean, near-shore conditions resulting from anthropogenic forces. The mostly aerobic *Flavobacteriaceae* family of microbes are physiologically similar to the *Pseudomonadaceae* and *Pseudoalteromonadaceae* families (Agronomique, Nakagawa, and Holmes 2002). However, the increased relative abundance of *Flavobacteriaceae* in captive metagenomes may reflect a reduced available nutrient as microbes utilizing slow growth to navigate starvation naturally outcompete copiotrophic rivals.

The epidermal microbiome alpha-diversity showed no significant differences across host environment, with less abundant taxa (<1%) accounting for little dissimilarity. Thus, we theorize the environment influences the relative abundance of the most abundant taxa while the hosts recruit taxon and maintain community diversity of epidermal microbiomes. Conversely, host factors such as biological sex, size, age, and pregnancy are known to play a role in shaping microbial associations the effect captive environments exert over major contributors to epidermal microbiomes suggests these and other host factors help maintain recurrent compositions that are worth exploring in future analyses. For example, the increased presence of *Moraxellaceae* in semi-captive sharks may reflect the transitional period from wild to captive lifestyles or a compounding effect of pregnancy as the semi-captive population was gravid during sampling. Therefore, while we have provided an important baseline representing the microbiomes of wild, semi-captive, and captive *T. semifasciata* populations, the gravid status of hosts may play a larger role in microbial community composition than the environment and individual host factors including age, sex, and weight should be investigated further.

Metabolic Potentials of Captive Shark Microbiomes Reflect Environmental Conditions

The epidermal microbiomes of captive *T. semifasciata* exhibited different metabolic potential although only at SEED subsystem level 2 and 3 and these higher-resolution variations are attributed to environmental differences. For example, captive *T. semifasciata* epidermal metagenomes had higher levels of several pathways involved in carbohydrate metabolism including mannitol utilization and fructose and mannose inducible phosphotransferase. While increased abundances of carbohydrate metabolism pathways in captive shark microbiomes are indicative of high levels of simple sugars in the environment, higher observed levels methylglyoxal pathway (MG) potential in captive metagenomes suggests excess nutrients available for captive microbes. The MG pathway produces aldehyde methylglyoxal instead of adenosine triphosphate, a toxic electrophile to cells. The MG pathway is therefore a low-energy-yielding glycolysis bypass that must be tightly regulated and is theorized to occur as a high-risk mechanism to harbor excess nutrients when catabolic rates increase, specifically sugar phosphate levels (Weber, Kayser, and Rinas 2005). Correspondingly, alcohol synthesis pathways i.e., acetone butanol ethanol synthesis gene pathway, metabolically follow carbohydrate metabolism and

were also elevated in captive samples. Adjacent to nutrient metabolism, genes (SEED subsystems: Level 3) annotated for mycolic acid synthesis, peptide methionine sulfoxide reductase, and chorismite synthesis were all measured higher in captive metagenomes, indicating higher rates of cell wall synthesis, protein processing, and amino acid synthesis respectively. From these findings we surmise microbes associated with captive sharks benefit from stable abiotic conditions in aquaria, utilizing an overabundance of nutrients and experiencing increased catabolism compared to wild counterparts as a result of scheduled aquaria feeding and supplement offerings.

While captive metabolic pathways indicated the amount of environmentally available carbohydrates exceed wild environmental levels, limited heavy metal availability was equally inferred. Several specific, key functions found to be significantly higher in captive samples revealed captive environments to have iron limiting conditions i.e. metabolic pathways such as iron acquisition, heme uptake and heme utilization indicate a need for bacteria to sequester iron when the heavy metal is not readily found in the environment (Gunn et al. 2019). Competitive microbial interactions in captive environments could also be inferred from increased bacteriophage and vibrio-related genes such as murein hydrolases i.e. hydrolytic enzymes known to cleave bacterial cell walls during final stages of the lytic cycle (Moak and Molineux 2004), and aspartate amino transferase i.e. enzymes key for bacteriophage infection (Chen et al. 2013). Last, shark microbiomes sampled in the captive environment displayed overall higher rates of protein misfolding within bacterial cell walls (Merdanovic et al. 2011), as seen in periplasmic stress gene. Overall, captive environmental conditions prompt higher rates of nutrient-hoarding, phage interactions, and heavy-metal sequestering among microbes associated with elasmobranch hosts. Further metagenomics studies should determine which factors besides captivity e.g., temperature, light, etc, also promote these effects. In addition, while this data was collected from metagenomics, these findings could be further explored by metatranscriptomics.

MAGs reveal Novel, Constant Microbial Associations with *T. semifasciata*

Construction of genomes confirmed several microbial associations with *T. semifasciata* in addition to metabolic contributions these microbes encode. For one such association, we confirmed a novel and continuous relationship with two bacteria belonging to the *Muricauda* genus associated with *T. semifasciata* across captive environments. Historically, this genus of marine microbes is genomically underexplored and free living, isolated from both seawater and marine sediments in Antarctica (Lee et al. 2012; S. Q. Liu et al. 2018; Yang et al. 2013; Zhang et al. 2018) and more recently from the gills of shrimp in Okinawa (L. Liu et al. 2020). Therefore, this is the first instance of *Muricauda* genus being associated with a vertebrate host organism and may be a shark-specific microbe. The associative driving force between the *Muricauda* genus and Chondrichthyes is not immediately apparent: while other deep-sea, marine bacteria are capable of utilizing trimethylamine N-oxide (TMAO; a well-known organic compounds used by sharks and other fish for osmotic regulation), as a carbon and nitrogen source, the *Muricauda* genus was shown to lack the necessary enzymatic activity to reduce the macromolecule (Yin et al. 2019).

Although some members of the *Bacteroidetes* phylum are characterized as opportunistic pathogens, the *Muricauda antarctica* species is deemed a low pathogen risk.

Additional MAG annotation belonging to the *Bacteroidetes* phylum was Bin 21, identified as *Roseivirga pacifica*. The *Roseivirga* genus has been isolated from seawater and sea urchins (*Strongylocentrotus intermedius*), an organism featured in benthic shark diets and a likely source of recruitment (Nedashkovskaya et al. 2005). The annotation of Bin 30 as *Zunongwangia atlantica* confirmed another microbe discovered in a deep-sea environment (Shao et al. 2014), and like the *Muricauda* genus, is also non-motile and strictly aerobic. Bin 22, identified as *Leeuwenhoekiella blandensis*, similarly aerobic, prefers warmer growth conditions (28-30° C; Pinhassi et al. 2006) and may be explained by aggregations of the sharks that occur in summer months in shallower waters.

While we were unable to annotate Bins 36 and 47 with confidence, the MAGs were found to contain the least contamination of the nine investigated MAGs. Uncharacterized MAGs are thought to be novel species as their sequences were highly complete. Future annotation of these genomes will no doubt reveal unique host-microbe associations and implications for host-microbe interactions. It should be noted the assembly of MAGs revealed a significantly larger contribution of contigs from wild samples, suggesting higher microbial loads in wild environments.

Conclusion

We investigated the associated microbial communities belonging to *T. semifasciata* housed since birth at the Birch Aquarium at Scripps Institution of Oceanography in La Jolla, a population held temporarily (<1 year) at the Scripps Institute of Oceanography, and wild populations in La Jolla and Moss Landing, California during summer months. We found these leopard shark epidermal microbiomes to be taxonomically distinct from respective environmental water columns with some inter-location variability. Our investigations into the impact of captivity on microbiome profiles revealed no significant differences in alpha-diversity indices, proportional abundance of taxonomy or function, and therefore captivity exert no detriment on biodiversity of shark-associated epidermal microbiomes.

We also measured the similarity of epidermal microbiomes community compositions belonging to *T. semifasciata* across environments to be the same, albeit with varied structures, as beta-diversity measures showed no significant difference between the groups. We also discovered as sharks are held in captivity for longer durations, their microbiomes proportionally deviate from wild hosts; our results show captive environments influence relative abundances of key generalist bacteria while hosts regulate the presence of microbes. Last, metagenome assembled genomes from *T. semifasciata* epidermal microbiomes identified and confirmed novel and consistent associations between the *Muricauda*, *Zunongwangia*, *Roseivirga* and *Leeuwenhoekiella* genera and the shark hosts. All shark groups contributed to the generation of the genomes (Figure 6), confirming the persistent presence of these microbes associated with *T. semifasciata*.

Declarations

Abbreviations:

Not applicable

Ethical Approval and Consent to participate:

Leopard shark samples was conducted under ethics approval from the SDSU ethics board APF 17-11-010D, APF 18-05-007D, APF # 014-05-011D, and APF # 11-02-004D. Birch aquarium sharks were sampled under the ethic approval by the Birch Aquarium ethic board.

Department of Fish and Game Scientific collecting permit: 9893, Dr. Andrew Nosal, Scripps Institute of Oceanography, secured June 4th, 2016. Department of Fish and Game Scientific collecting permit: 12847, Dr. Michael Doane, Secured November 30th, 2014.

Consent for publication:

Not applicable

Availability of supporting data:

All data will be publicly released upon publication

Competing interests:

The authors declare no conflict of interest.

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Authors' contributions:

AG collected field samples, analyzed the data, and wrote the manuscript, NP conducted bioinformatics, MD collected field samples, and constructed the ideas, CJ, EK, MM, captured sharks and prepared samples and provide feedback on the manuscript, LL and EK provided sequencing and analysis, AN facilitated the catching of leopard sharks and access to leopard sharks are Scripps Research Facility, MT and JN provided support for sampling Birch Aquarium Sharks and the design of the experiment, ED conceptualized the idea, analyzed, wrote and lead the project.

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Figures

Figure 1

Sampling of the flank region on *T. semifasciata* (A) with a closed-circuit, blunt faced syringe tool (B) used for the collection of shark skin microbiome samples.

Figure 2

Variations among epidermal microbiome taxonomic composition belonging to *T. semifasciata* across captivity status. Height of bars represent relative abundance of most abundant phyla (>1%) at family level.

Figure 3

Non-metric dimensional scaling of Bray-Curtis similarity between epidermal microbiomes belonging to *T. semifasciata* across environments. Weak community dissimilarity observed between wild and captive populations at family level, with taxa influencing spatial groupings overlaid (>0.85 correlation). Relative abundance is normalized, fourth root transformed, and analysis is corrected with Bonferroni.

Figure 4

Significant differences in gene potentials (SEED Subsystem: Level 2) observed in epidermal microbiomes associated with *T. semifasciata*. Clustering dendrogram above heatmap orients nearest neighbor clustering of *T. semifasciata* metagenomes grouped by host environment and captivity status: long-term captive individuals (Birch Aquarium, blue), short-term captive individuals (Scripps Institute of Oceanography, purple), and wild individuals (green). Variations of relative functional gene abundances are visualized using a bi-colored heatmap from higher (blue) to lower (white) functional gene abundance.

Figure 5

Percent distribution of functional pathways within epidermal microbiomes belonging to *T. semifasciata* across environments. Systematic top ten percent of reads representing specific (SEED subsystems: Level 3) gene pathways significantly differing between epidermal microbiomes belonging to captive, semi-captive, and wild *Triakis semifasciata* across environments.

Figure 6

Phylogram depicting mean coverage of MAGs, ordered by total reads mapped. Highlighted groups aid visualization of even contribution across captive environments in three groups, and location specific MAGs in gray.

Figure 7

Pangenome frequency of *Muricauda antarctica* compared to MAG Bins 9 and 27. The clustering dendrogram to the right of the circular phylogram displays the hierarchical clustering of contigs based upon their differential sequence coverage across MAGs and the *Muricauda antarctica* reference genome (light blue). Below the dendrogram are genome indices including completion (%), number of contributing genes per 1 kbp for each open reading frame found, the redundancy level, and GC-content. Each sample layer (*Muricauda antarctica*, Bin 27, and Bin 9) represents gene cluster frequency, ordered by the combined homogeneity index auxiliary layer. Additional auxiliary layers from outside in, following

combined homogeneity index, represent the number of contributing genomes and single copy gene clusters (SCG).

Supplementary Files

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