The Gills of Reef Fish Support a Distinct Microbiome Influenced by Host-Specific Factors

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ABSTRACT Teleost fish represent the most diverse of the vertebrate groups and play important roles in food webs, as ecosystem engineers, and as vectors for microorganisms. However, the microbial ecology of fishes remains underexplored for most host taxa and for certain niches on the fish body. This is particularly true for the gills, the key sites of respiration and waste exchange in fishes. Here we provide a comprehensive analysis of the gill microbiome. We focus on ecologically diverse taxa from coral reefs around Moorea, sampling the gills and intestines of adults and juveniles representing 15 families. The gill microbiome composition differed significantly from that of the gut for both adults and juveniles, with fish-associated niches having lower alpha diversity values and higher beta diversity values than those for seawater, sediment, and alga-associated microbiomes. Of ~45,000 operational taxonomic units (OTUs) detected across all samples, 11% and 13% were detected only in the gill and the intestine, respectively. OTUs most enriched in the gill included members of the gammaproteobacterial genus *Shewanella* and the family *Endozoicimonaceae*. In adult fish, both gill and intestinal microbiomes varied significantly among host species grouped by diet category. Gill and intestinal microbiomes from the same individual were more similar to one another than to gill and intestinal microbiomes from different individuals. These results demonstrate that distinct body sites are jointly influenced by host-specific organizing factors operating at the level of the host individual. The results also identify taxonomic signatures unique to the gill and the intestine, confirming fish-associated niches as distinct reservoirs of marine microbial diversity.

IMPORTANCE Fish breathe and excrete waste through their gills. The gills are also potential sites of pathogen invasion and colonization by other microbes. However, we know little about the microbial communities that live on the gill and the factors shaping their diversity. Focusing on ecologically distinct types of coral reef fish, we provide a comprehensive analysis of the fish gill microbiome. By comparison to microbiomes of the gut and the surrounding environment, we identify microbes unique to the gill niche. These microbes may be targets for further studies to determine the contribution of the microbiome to waste exchange or host immunity. We also show that despite exhibiting a unique taxonomic signature, the gill microbiome is influenced by factors that also influence the gut microbiome. These factors include the specific identity of the host individual. These results suggest basic principles describing how association with fishes structures the composition of microbial communities.

KEYWORDS intestine, gut, bacteria, teleost, coral reef, Moorea

Teleost fish are among the most biodiverse of coral reef inhabitants, representing an estimated 6,000 to 8,000 total reef species (1). Reef fishes engage in complex energy and material transfer processes involving a range of trophic and symbiotic

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interactions, while also serving as reservoirs and vectors of microorganisms (2, 3). Such diversity makes reef fish communities useful for exploring the factors shaping animal-associated microbiomes (4). For fishes in general, it remains unclear how microbiome diversity is partitioned among niches, notably between the fish intestine and other body sites. In particular, our understanding of the mucosal microbiome of the fish gill remains limited. Given the role of the gills as a primary site for gas and waste exchange in fishes, as well as for mucosal immune interactions, osmoregulation, and detoxification (5), gill-associated microbiomes may play critical roles in overall fish health and physiology. In the present study, using reefs as a model system for sampling broadly across host diversity, we explore patterns of organization in the fish gill microbiome.

Animal-associated microbiomes often contain hundreds of bacterial or archaeal species, many of which are hypothesized to contribute to host health, behavior, and development (6, 7). These contributions are principally mediated by microbial chemical transformations, including the breakdown of host food and waste products and the synthesis of molecules that interact directly with host cells (8). These chemical transformations and the genetic diversity underlying these processes are becoming better understood for the microbiome of the vertebrate gastrointestinal (GI) tract, primarily the large intestine, where the vast majority of microbiome biomass is located (9). However, microbes exist in diverse niches throughout the animal body, including in association with non-GI mucosal surfaces that also function in chemical or gas exchange and as barriers or entry points for pathogens. Characterizing non-GI mucosal microbiomes—including those of the gills, lungs, and skin—is therefore critical for identifying novel functions of animal-associated microbes and for understanding the connectivity between environmental microbial communities and those of the host.

In fish, the mucosal surface of the gills may be a relatively unique habitat for host-associated microbes. The fish gill is potentially influenced by high local concentrations of oxygen and nutrients (e.g., ammonia and urea [10]), in contrast to the GI tract, where oxygen may be more limiting and for which the genomic potential for anaerobic fermentative metabolism has been demonstrated (3); however, variation in intestinal oxygen availability across fishes with differing dietary ecologies and GI structures is not well understood. Furthermore, the gills may undergo high levels of mucosal discharge, especially during times of environmental stress (e.g., sediment loading [11]), may exhibit high connectivity with the surrounding water microbiome and between the water and bloodstream (5), and may be important access points for pathogens (12). The extent to which the gill community is more or less influenced by colonization by external microbes than are microbiomes of other body site niches, such as the GI tract, remains uncertain; indeed, the fish intestinal microbiome is presumably also highly connected to the environmental microbiome via the intake of food as well as seawater, which saltwater fish regularly “drink” to help maintain water balance. Together these factors suggest a need for further work to explore the gills as sites for unique assembly processes or novel microbe-microbe or microbe-host interactions, potentially involving symbioses. For example, the gill microbiome of rainbow trout was shown to have antifungal properties (13), and a recent study of carp gills identified intracellular bacterial symbionts that oxidize host-excreted ammonia as an energy source (14), thereby removing a potential toxin that can cause tissue damage at high concentrations (15). However, while the gill microbiome is likely distinct from that of the surrounding environment (e.g., see reference 16), the full diversity of the gill microbiome, the factors structuring this diversity, and the extent to which this diversity is specific to the gill or a host individual remain unknown.

Focusing on a single reef system around the Island of Moorea in French Polynesia, thereby minimizing confounding effects of geography, we present a comprehensive analysis of gill microbiome diversity across diverse reef fish taxa. We sampled fishes representing 15 families, diverse diet ecologies, and both juveniles and adults to test for a shared gill microbiome structure and the extent to which this structure varies from those of the intestinal microbiome and the microbiome of the surrounding environ-

TABLE 1 Sample numbers (*n*) and OTU statistics

Sample type	<i>n</i>	No. of OTUs ^a	No. (%) of unique OTUs ^b	Fish families (<i>n</i> individuals per family)
All samples (fish + environmental)	552	45,267	NA	
Fish samples (gill + intestine)	399	20,980	12,996 (29)	
Gill (adult + recruit)	187	13,480	4,901 (11)	
Gill (adult)	141	11,607	3,844 (9)	Acanthuridae (23), Blenniidae (6), Chaetodontidae (35), Cirrhitidae (12), Holocentridae (12), Labridae (5), Lethrinidae (2), Lutjanidae (4), Mullidae (4), Pomacanthidae (5), Pomacentridae (19), Scaridae (4), Scorpaenidae (6), Serranidae (4)
Gill (recruit)	46	4,119	863 (2)	Chaetodontidae (8), Holocentridae (34), Pomacanthidae (4)
Intestine (adult + recruit)	212	12,709	5,835 (13)	
Intestine (adult)	176	11,519	5,380 (12)	Acanthuridae (25), Balistidae (4), Blenniidae (7), Chaetodontidae (31), Cirrhitidae (21), Holocentridae (13), Labridae (3), Lethrinidae (1), Lutjanidae (6), Mullidae (4), Pomacanthidae (5), Pomacentridae (36), Scaridae (6), Scorpaenidae (4), Serranidae (10)
Intestine (recruit)	36	2,255	400 (1)	Chaetodontidae (9), Holocentridae (20), Lutjanidae (1), Pomacanthidae (6)
Environmental samples (all)	153	32,223	24,239 (54)	
Seawater	37	6,505	2,212 (5)	
Sediment	38	16,864	9,820 (22)	
Algae	68	17,773	8,978 (20)	Cladophoraceae (2), Codiaceae (4), Chordariaceae (6), Dictyotaceae (15), Sargassaceae (21), mixed turf (20)
Coral	10	862	102 (0.2)	Poritidae (10)

^aOTUs are 97% sequence similarity clusters. Totals are the sums of per-sample counts (based on a depth of 3,000 sequences per sample). NA, not applicable.

^bOTUs found only in a given sample type compared against all other fish and all other environmental samples.

ment. These analyses identify key microbial taxa specific to the gill niche and also provide support for the hypothesis that the gill and intestinal microbiomes are influenced by similar host-specific organizing factors, whose strengths vary with life stage.

RESULTS

Microbiome samples. We analyzed 552 microbiomes from Moorea, French Polynesia (see Fig. S1 in the supplemental material), including 399 fish microbiomes representing the gills of 141 adults and 46 juvenile recruits and the intestines of 176 adults and 36 recruits. An additional 50 gill and 97 intestine samples were collected but failed to yield visible PCR products. Fish microbiome samples spanned 53 species within 15 families in the order Perciformes (Table 1; Fig. 1), with each family represented by 1 to 36 individuals. Based on the literature, the sampled taxa are roughly equally distributed among the three diet categories of carnivore, herbivore, and omnivore (Fig. 1; Table S1). However, some taxa likely do not adhere strictly to a single diet category; a comprehensive assessment of fish microbiome structuring based on diet should therefore involve a coupled assessment of diet contents.

The nature of the field collections did not allow recovery of the same set of host taxa between spearfished (adult) and crest net-collected (recruit) sample sets. While adult individuals were obtained for all 15 families, recruits were obtained only for the Chaetodontidae, Holocentridae, Lutjanidae, and Pomacanthidae families (Table 1). Therefore, comparisons of microbiomes between adults and recruits should be interpreted with caution, as differences may be due to changes in host factors, such as fish age, or to differences in host taxonomic composition.

We therefore emphasize comparisons between the gill and intestinal niches within a life stage, as the taxonomic representation of host individuals was relatively uniform between these niches within a life stage (Table 1). These results are presented below and are contextualized by comparison to microbiomes of seawater, sediment, algae, and coral from around Moorea ("environmental" samples in Table 1 [*n* = 153]).

Niche-specific structuring of 16S rRNA gene sequence diversity. Microbiome diversity (alpha and beta) varied significantly between animal-associated and environmental samples. In total, we recovered ~45,000 microbial operational taxonomic units (OTUs; 97% similarity clusters) across all samples after rarefaction to a common se-

FIG 1 Alpha and beta diversity values for all fish samples, organized by host phylogenetic affiliation and presumed host diet type. The phylogeny is based on the COI gene, with the size of gray circles indicating bootstrap values of >0.80 . Alpha diversity is expressed as Chao1 richness estimates, as determined by QIIME. Beta diversity is expressed as dispersion, the distance from individual samples to the centroid of the sample type, as determined by using the PERMANOVA function in PRIMER 7.

quence count (Table 1). Of these, 11% and 13% were detected only in fish gills and intestines (recruit or adult), respectively. On average, of the OTUs detected in fish-associated niches, 75 to 85% were also detected in environmental samples (Table S2). Of the environmental samples, sediment and alga samples contributed the highest percentages of unique OTUs (22% and 20%, respectively), despite being represented by considerably fewer samples. These results are consistent with fish gill and intestinal microbiomes having significantly lower Chao1-estimated OTU richness values than those of microbiomes of seawater, algae, and sediment ($P < 0.01$; Tukey *post hoc* test) (Table S3), which were, on average, 3 to 11 times higher (Fig. 2). Rather, fish microbiome richness was comparable to that of the coral (*Porites*) microbiome. On average, Chao1 estimates for intestinal samples were comparable to those reported previously (2, 17, 18). Despite their high level of richness, environmental microbiomes of algae, water, and sediment clustered according to sample type based on a nonmetric multidimensional scaling (NMDS) analysis of Bray-Curtis dissimilarity (OTU level) (Fig. 3). In contrast, fish-associated microbiomes exhibited high intersample variation (Fig. 2 and 3; Fig. S2), notably with those of adult fish (both gill and intestine) having significantly higher values for dispersion (distance to the centroid for each sample type grouping) than those of all other microbiome types, excluding those of the recruit intestine ($P < 0.01$; Tukey *post hoc* test) (Table S4).

Focusing on the fish-associated samples, we observed significant differences in microbiome structure based on body site. While microbiomes of the four fish sample types exhibited comparable levels of alpha diversity (Fig. 2; Table S3), with the exception of those of the recruit intestine, analysis of similarity (ANOSIM) based on Bray-Curtis matrices revealed that the taxonomic composition differed significantly between the gill and the intestine for both recruit and adult data sets ($P < 0.01$; ANOSIM) (Table 2; see Fig. 5). The magnitude of dissimilarity between gill and intestinal microbiomes was greater for recruits (ANOSIM global $R = 0.47$) than for adults ($R = 0.11$) (Table 2), although we caution that comparison across life stages is confounded by differences in host taxonomic composition.

FIG 2 Microbiome alpha and beta diversity values. Alpha diversity is shown as Chao1 estimates of OTU richness, with box plots showing the 25th to 75th percentiles and whiskers showing a maximum of $1.5 \times$ the interquartile range (IQR). Intersample variation (beta diversity) is shown as dispersion, with the y axis indicating the distance from each sample to the sample type centroid based on Bray-Curtis distances. Both OTU richness and dispersion varied significantly among sample types ($P < 0.001$ by ANOVA); results of pairwise Tukey *post hoc* tests for significant variation between sample types are shown in Tables S3 and S4 in the supplemental material.

Gill and intestinal microbiomes also differed in the extent to which they were structured based on presumed host diet. For both recruit and adult fish, the intestinal microbiome composition varied significantly among carnivores, herbivores, and omnivores ($P < 0.05$; ANOSIM) (Table S5). However, diet type-specific microbiome clustering was most pronounced for the adult intestine (Fig. 4). Among the intestinal samples, the microbiomes of adult carnivorous fish showed the tightest clustering, with ANOSIM R values of 0.52 and 0.32 for comparisons against herbivore and omnivore microbiomes,

FIG 3 Similarities in composition among Moorea microbiomes. Clustering is based on nonmetric multidimensional scaling analysis of all microbiome samples according to Bray-Curtis distances evaluated at the OTU level (97% sequence similarity).

TABLE 2 One-way ANOSIM results for comparisons of gill and intestinal microbiomes of recruit and adult fish

Comparison	Global <i>R</i> value	<i>P</i> value
Adult gill vs adult intestine	0.109	0.001
Adult gill vs recruit gill	0.362	0.001
Adult intestine vs recruit intestine	0.240	0.001
Recruit gill vs adult intestine	0.154	0.001
Recruit gill vs recruit intestine	0.465	0.001
Recruit intestine vs adult gill	0.579	0.001

respectively ($P < 0.01$) (Table S5), whereas the latter microbiomes were more similar to one another ($R = 0.13$). Interestingly, adult gill microbiomes were also significantly partitioned according to diet type ($P < 0.01$), although the extent of this structuring was less than that for the adult intestine samples (global R values of 0.22 and 0.35 for gill and intestine comparisons, respectively). Significant structuring based on diet type was not observed for the gill microbiomes of recruits (Table S5).

Analyses of paired data sets (gill and intestinal microbiomes from the same individual) provided evidence that gill and intestinal microbiomes were influenced jointly by the same host-specific assembly factors. Using data from all adult individuals for which paired data sets were available ($n = 110$), the average Bray-Curtis similarity between gill and intestinal microbiomes within an individual was significantly greater (>2 -fold) than that between gill and intestinal microbiomes from different individuals ($P < 0.001$) (Table 3), with gill and intestine data sets from the same individual sharing,

FIG 4 Similarities in composition among Moorea microbiomes grouped according to presumed host diet category. Clustering is based on nonmetric multidimensional scaling analysis of all microbiome samples according to Bray-Curtis distances evaluated at the OTU level (97% sequence similarity). Host diet category designations are based on the data in Table S1 in the supplemental material.

TABLE 3 Similarities between gill and intestinal microbiomes

Fish group (n)	Mean Bray-Curtis similarity value ^a		P value
	Within individual	Between individuals	
Adults			
All taxa (110)	19.6	9.6	<0.001
Chaetodontidae (27)	22.2	12.3	0.003
Pomacanthidae (5)	21.9	21.5	0.97
Holocentridae (10)	19.5	17.2	0.44
Recruits			
All taxa (24)	21.2	17.3	0.08
Chaetodontidae (6)	16.2	14.1	0.67
Pomacanthidae (3)	33.3	23.0	0.31
Holocentridae (15)	20.7	16.9	0.07

^aPotential values range from 0 to 100, with higher values indicating higher levels of similarity.

on average, ~24% of OTUs (Table S2). Significantly higher similarity within versus between individuals was also observed for adults of the Chaetodontidae. Consistently higher similarity within versus between individuals was observed for all other comparisons, including those involving recruits; however, these differences were not significant, potentially due to smaller sample sizes (Table 3).

Gill-associated taxa. The taxonomic signature of the gill microbiome was distinct from that of the intestinal microbiome. Analysis of microbial taxa at the class/subclass level or above showed that gill and intestinal microbiomes share many of the same broad taxonomic groups, with both niches dominated by members of the *Gammaproteobacteria* (Fig. 5), and that there is a high level of variation among gill and intestinal microbiomes (Fig. S2). However, in both adults and recruits, gill microbiomes were enriched (with higher proportional abundances) in members of the *Betaproteobacteria* (5.9- to 72.0-fold), *Flavobacteria* (2.0- to 5.9-fold), and *Saprospirae* (2.8- to 11.9-fold) compared to intestinal microbiomes. In contrast, in both life stages, intestinal microbiomes were enriched (5.8- to 185.9-fold) in *Brevinematae*, members of the *Spirochaetes* reported for microbiomes of both aquatic and terrestrial animals (17, 19).

These broad patterns are supported by analysis at finer taxonomic levels. DESeq2 tests revealed 177 microbial OTUs significantly enriched in the gill microbiome (recruit and adult data combined) compared to all other data sets (Table 4; Table S6). The most enriched taxa, based on changes in mean proportional abundance, included an unidentified betaproteobacterial OTU with highest similarity to unclassified environmental clones from diverse aquatic habitats (data not shown) and multiple OTUs of the gammaproteobacterial genus *Shewanella* and the family *Endozoicimonaceae* (Table 4). Several of these groups were highly prevalent. Notably, one of the *Endozoicimonaceae* OTUs was detected in 81% of all adult gill samples, at an average abundance of 4%, but in only 14% of adult intestine samples, at an average abundance just above the detection limit (Table S7). In contrast, the OTUs most enriched in the intestine compared to the gill included multiple unclassified members of the *Brevinemataceae* and the family *Pirellulaceae* of the phylum *Planctomycetes* (Tables S8 and S9). The most intestine-enriched OTUs included a member of the genus *Epulopiscium* (*Firmicutes*), a potential fish symbiont linked to algal polysaccharide degradation in reef surgeonfish (3), as well as many diverse phototrophic cyanobacterial lineages, presumably reflecting cells consumed as or in association with food.

DISCUSSION

The gills of reef fish host a unique and complex microbiome. Analysis of 53 fish species common to South Pacific reefs confirmed that the microbiomes of the gill and intestine are distinct from one another and from those of surrounding environmental reservoirs. Like the fish intestine and corals, the gill contains significantly fewer OTUs than those in the surrounding seawater and nearby sediment and algae. This is

FIG 5 Average microbiome taxonomic compositions of all sample types. Taxa were grouped at the class/subclass level by using a rarefied OTU table (3,000 sequences per sample). All 239 detected classes were present in either gill or intestine samples and at least one environmental sample (coral, algae, sediment, or water). The bar plot shows the log₂ fold change in mean proportional abundance between gill and intestine data sets. The microbiome taxonomic compositions of individual fish are shown in Fig. S2 in the supplemental material.

consistent with recent comparisons showing decreased diversity in animal-associated microbiomes compared to that in free-living microbiomes (20). Indeed, the vast majority of gill and intestine OTUs were also detected in environmental samples (Table 1; see Table S5 in the supplemental material), suggesting that both body niches select for a nested subset of the external community.

However, the gill also includes potentially niche-specific members. Of the >45,000 OTUs recovered across all data sets, ~10% were detected only in the gill samples. Of these, 177 OTUs were significantly enriched in the gill compared to all other sample types. The fish gill is particularly enriched in certain members of the gammaproteobacterial genus *Shewanella* and the family *Endozoicimonaceae*. *Shewanella* species are metabolically diverse and common members of free-living microbial communities in diverse aquatic habitats and of fish microbiomes, including those in the intestine and in association with disease (2, 18, 21, 22). The most enriched *Shewanella* OTU (814252) (Table 4; Table S6) was 100% identical to an environmental sequence recovered from Pacific herring (GenBank accession number [JQ191540.1](#)) and 99% identical to the common fish associate *Shewanella putrefaciens* and the potential human pathogen *Shewanella alga*. Endozoicomonads have been recovered ubiquitously from mucosal surfaces of marine invertebrates, particularly corals (23, 24), but have been reported less commonly for fish (23). In the present study, the three most enriched endozoicomonad OTUs share 99 to 100% identity with sequences recovered from diverse marine hosts, with the most enriched OTU (370251) (Table 4; Table S6) being 100% identical to a putative symbiont isolated from the coral *Montipora* (25). Although studies of aquacultured fishes implicate endozoicomonads in diseases of the fish gill (26, 27), our results suggest that this group is a stable and enriched component of the fish gill niche.

TABLE 4 Most enriched OTUs in gill microbiomes^a

Phylum	Class	Order	Family	Genus	Species	Change ^b	Relative abundance ^c
Proteobacteria	Betaproteobacteria	NA	NA	NA	NA	9.4	1.21 ± 7.6
	Gammaproteobacteria	Legionellales	Francisellaceae	NA	NA	7.5	0.59 ± 2.7
	Gammaproteobacteria	Oceanospirillales	Endozoicimonaceae	NA	NA	6.3	5.66 ± 13.6
	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	NA	5.8	2.24 ± 6.7
	Gammaproteobacteria	Oceanospirillales	Endozoicimonaceae	NA	NA	5.7	0.08 ± 0.4
	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	NA	5.1	0.29 ± 1.4
	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	NA	4.8	0.19 ± 0.9
	Alphaproteobacteria	Rickettsiales	NA	NA	NA	4.8	0.22 ± 2.5
	Gammaproteobacteria	Oceanospirillales	Endozoicimonaceae	NA	NA	4.8	1.44 ± 6.2
	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	NA	4.5	1.36 ± 2.7
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA	4.5	0.95 ± 3.3
	Alphaproteobacteria	Rickettsiales	Anaplasmataceae	Neorickettsia	NA	4.4	0.08 ± 0.4
	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	NA	4.4	0.05 ± 0.1
	Alphaproteobacteria	Rhizobiales	Bartonellaceae	NA	NA	4.3	0.06 ± 0.2
Bacteroidetes	Cytophagia	Cytophagales	"Amoebophilaceae"	NA	NA	4.2	0.05 ± 0.2
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA	4.2	1.69 ± 6.8
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	NA	4.1	0.03 ± 0.1
Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium	NA	4.1	1.07 ± 2.9
Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	Wolbachia	NA ^d	4.1	0.70 ± 1.8
	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	NA	4.0	0.05 ± 0.1

^aThe OTUs listed are 16S rRNA gene 97% similarity clusters identified as significantly differentially abundant by analysis of all fish gill data sets versus all other data sets (adult and recruit fish intestine and environmental samples) by use of DESeq2. OTUs are ranked by fold change. Complete lists of all gill-enriched and intestine-enriched OTUs can be found in Tables S6, S8, and S9 in the supplemental material. NA, not available.

^bLog₂ fold change in mean proportional abundance.

^cMean proportional abundance (%) across all fish gill samples (± standard deviation [SD]), based upon the rarefied (3,000 sequences) OTU table.

^dWolbachia endosymbiont of *Chorthippus parallelus*.

Other gill-enriched OTUs include groups previously associated with external surfaces of fish, including the skin and fins. These include *Shewanella*, *Cetobacterium*, and *Clostridium* (28, 29) as well as representatives of the obligately intracytoplasmic genus *Neorickettsia* (30), which has been implicated in fish-transmitted disease in mammals (31), and the methylotrophic genus *Methylobacterium*, which has been suggested to be a fish commensal with a role in beneficial antibacterial activity (32). This suggests that the gill microbiome likely resembles those of other exterior habitats of the fish body. Like that of the gills, the skin and fin microbiomes are also associated with mucus and presumably shaped by high connectivity to the external environment. Indeed, the microbiome composition of the skin mucus has been used as a marker of harvest location for commercial food fish (33, 34), and sampling date and location have been shown to play a significant role in structuring the microbiome of the dorsal fin (30). In the present study, the high percentage of gill OTUs shared with the environmental samples (>75%) (Table S2) indicates that the gill microbiome may be influenced similarly by variation in the surrounding free-living microbial community.

It is also possible that the gill-specific microbiome is influenced by the unique functions of the gills in waste excretion, gas exchange, and body site-specific immune activity (35). For example, the degree to which the microbiome interacts with fish immune factors can differ between the skin and gills (36). Also, ammonia excretion in fish occurs primarily through the gills (37) and may select for ammonia-utilizing microbes (14). In the present study, the set of OTUs enriched in the gill niche did not include taxonomic groups canonically associated with ammonia oxidation (Table S6). However, analysis involving only the gill and intestine data sets (no environmental data sets) identified two OTUs of the ammonia-oxidizing *Thaumarchaeota* among the taxa most enriched in the gill compared to those most enriched in the intestine (Table S8). We caution, however, that the metabolic functions of the gill microbiome remain essentially unknown. Exploring these functions directly through metagenomics, physiological assays of gill tissue, or culture of the dominant gill-enriched microbes identified here is necessary to identify the chemical and ecological processes unique to the gill niche.

Shared assembly processes. Despite clear taxonomic differences, gill and intestinal microbiomes show similarities suggesting that these niches are influenced partly by the same organizing factors. Both niches appear to be structured jointly by processes operating at the level of the host individual. In recruits and adults and across different fish families, the gill microbiome of an individual, on average, shares 20 to 25% of its OTUs with the microbiome of the intestine (Table S2) and is more similar to the intestinal microbiome of the same individual than to that of a different fish (Table 3). This pattern can be explained if the gill microbiome exchanges members with the microbiomes of other body sites, potentially via the bloodstream. Indeed, a study of wild-caught snapper showed broad similarity between gill and blood microbiomes (38). Gill and intestinal microbiomes of the same fish may also be similar because they were colonized at the same time from the same free-living pool (in contrast to other individuals) or are jointly influenced by systemic host factors operating across body sites, potentially including the chemical or immune cell composition of host mucus (39–41). Indeed, microbes interact with the immune system in both the gills and the intestine, and the coating of microorganisms by certain immunoglobulins has been shown to be higher in these niches than at other microbiome sites (36, 42). This suggests the occurrence of similar immune interactions at these sites and raises the possibility of similar selections for specific microbes. However, it should be noted that our study could not distinguish between microbes that maintain longer-term residence in the host and more transient (allochthonous) members of the microbiome. It is possible that if they were distinguished, these two communities would exhibit differential patterns of relatedness across body niches (gill and intestine); this topic deserves further study.

For adult individuals, fish microbiome composition varied among host species grouped by diet category. This effect was greatest in the adult intestine, consistent with a recent analysis of freshwater fishes showing gut microbiome structuring based on trophic level (22). Interestingly, the gill microbiome of adults was also significantly, though much more subtly, influenced by diet category. This is consistent with the high beta diversity observed for both gill and intestine data sets from adult fish (Fig. 2 and 3). We caution, however, that diet is confounded by host phylogenetic identity (Fig. 1), and the high level of microbiome structuring in adult fish is potentially due to sampling across a wide range of diverse host types, each of which covaries with a range of variables (e.g., habitat use and host physiology) in addition to diet. Indeed, a prior study examining Atlantic salmon reared on various diets revealed no effect of diet variation on gill microbiome structure (43). In our study, microbiome composition also varied significantly among adult microbiomes partitioned according to host family (data not shown). Thus, although the data confirm a general effect of host specificity on gill microbiome assembly, we cannot definitively link this effect to variation in diet.

Although comparisons between recruit and adult samples should be interpreted with caution, as these two categories were not represented by exactly the same host taxa, the overall trends in this study suggest that processes of gill microbiome structuring may vary with life stage. In comparison to those of adult fish, the gill and intestinal microbiomes of juvenile recruits were less likely to vary significantly among hosts grouped by diet type (Fig. 4; Table S5). This is consistent with a stronger role for stochastic colonization of the microbiome in young fish, followed by enhanced selection for host-specific assemblages as fish age. A study of captive zebrafish showed that nonneutral processes, such as selection for the host environment, play a more dominant role in microbiome assembly in adults than in juveniles (44). This is perhaps not surprising, as the microbiome is presumably responsive to many of the processes that occur as fish age, including immune system development, increased specialization of the host diet, and exposure to different external environments (e.g., off reef versus on reef). Indeed, a prior study of wild-caught damselfish and cardinalfish indicated a significant shift in intestinal microbiome composition over the transition from pelagic larvae before reef settlement to postsettlement juveniles and adults (2). This transition

may involve shifts in diet (45). Indeed, we know less about the diets of young fish, and our diet category designations were inferred from studies focused predominantly on adults. If microbiome assembly pressures change markedly over the host life span, then microbiome compositional variation between body sites may be less than the variation in the same body site sampled at different host life stages. In the present study, gill microbiomes of adults were more similar to intestinal microbiomes of adults ($R = 0.11$ by ANOSIM) than to recruit gill microbiomes ($R = 0.36$). This is intriguing given the presumably large differences in the chemistries of the gill and intestinal niches, notably with the latter influenced by incoming food. Together the data confirm that host factors influence the gill microbiome but likely vary over time, with a potentially greater overall effect on microbiome assembly in adult individuals.

Conclusions. The reef fish gill microbiome appears to be organized by a combination of stochastic and deterministic processes, including connectivity with the surrounding environment and host factors that vary with fish species and life stage, potentially including diet. Our results for paired gill and intestine data sets from the same individual provide the strongest evidence for microbiome organizing processes shared across body sites and also suggest a significant role for interindividual effects on microbiome assembly. Nonetheless, several microbial OTUs appear to be enriched consistently in the gill compared to all other studied niches. The results suggest these taxa as targets to identify microbial functions potentially specific to the gill niche. Although beyond the scope of this study, mining of these data sets, which span a broad spectrum of fish types, may also identify microbial taxa significantly associated with specific host types or ecological strategies. Thousands of microbial OTUs were detectable only in the fish gill or intestine, confirming these fish-associated niches as distinct reservoirs of marine microbial diversity. Understanding this diversity will inform our understanding of fish health and ecology as well as basic principles describing how host association structures microbiome assembly.

MATERIALS AND METHODS

Collection. Between 23 January and 15 February 2016, 207 adult fish and 55 juvenile recruits spanning 15 families and 53 species (Table 1; Fig. 1) were collected from 6 sites (see Fig. S1 in the supplemental material) around the island of Moorea, French Polynesia, via spear gun and hand nets (adults) or via crest net (recruits and some adults) (see references 46 and 47 for full descriptions of crest net methods). The exact capture locations of individual fish were not recorded. We therefore cannot discount the possibility of local variation in the water column microbiome as a determinant of gill/intestinal microbiome diversity; however, the relatively low beta diversity of water column microbiome samples from the different collection sites (Fig. 2) argues against a strong intersite effect.

All fish specimens were immediately euthanized either by pithing or by use of a lethal dose of tricaine mesylate (MS222) and placed directly on ice in coolers until arrival at the CRIOBE Research Station (typically 2 h later, and no longer than 6 h later). Each fish was measured (fork length by height) and photographed for identification (described below). Specimens were either dissected within ~12 h of capture (kept at 4°C or below until dissection), with most dissections performed within 2 to 5 h, or frozen at -20°C until dissection. All specimens were dissected in trays wiped with 100% ethanol. Due to their small size, which limited sample processing in the field, recruits were sorted according to species and date and frozen whole in 15-ml conical tubes filled with RNA/DNA stabilizing buffer (25 mM sodium citrate, 10 mM EDTA, 5.3 M ammonium sulfate, pH 5.2) until dissection at Georgia Tech. For both recruits and adults, either the entire gill or a gill fragment was dissected and placed in a cryovial, which was then filled with RNA/DNA stabilizing buffer. For each adult individual, we also dissected the entire intestine, which in teleost fish consists of the small intestine, where the majority of food digestion takes place, followed by the rectum; teleost fish lack the true large intestine present in other vertebrates. Adult fish intestines were strung out and cut in half, and the proximal half of each was preserved in a cryovial as described above. In adult *Ctenochaetus striatus*, *Chlorurus sordidus*, and *Scarus psittacus* fish, the intestinal lining was too thin for dissection without rupture. For these taxa, the entire intestine was used for analysis. Similarly, for recruits, the entire intestine was dissected and preserved in RNA/DNA stabilizing buffer in a cryovial to provide enough material for DNA extraction. All dissected and preserved samples were immediately placed at -20°C and were transferred to -80°C within 24 h.

Samples of seawater, sediment, coral, and dominant reef alga species (*Chaetomorpha* sp., *Codium* sp., *Hydroclathrus* sp., *Padina* sp., *Sargassum* sp., and filamentous turf) were also collected from each sampling site to characterize the microbiome of the surrounding environment. These samples are collectively termed “environmental samples” throughout this study. Three to 10 samples were collected per environmental sample type per site. For analysis of seawater microbiomes, ~0.5 liter of water was collected by bottle during dives (scuba or free). Bottles were placed on ice until return to the research station, where the water was filtered through a 0.2-μm Isopore membrane filter (Millipore). Filters were

TABLE 5 Primers and associated sequences

Primer	Sequence (5'–3')	Reference(s)
F515	GTGCCAGCMGCCGCGGTAA ^a	53, 54
R806	GGACTACHVGGGTWTCTAAT ^a	53, 54
C_FishF1t1		
VF2_t1	CAACCAACCACAAAGACATTGGCAC ^b	49
FishF2_t1	CGACTAATCATAAAGATATCGGCAC ^b	49
C_FishR1t1		
FishR2_t1	ACTTCAGGGTGACCGAAGAATCAGAA ^b	49
FR1d_t1	ACCTCAGGGGTGCCGAARAAYCARAA ^b	49

^aIndex and adapter sequences are not shown.^bM13 tails are not shown.

immersed in RNA/DNA stabilizing buffer in cryovials and frozen. Samples of sediment were scooped (without touching by hand) into sterile 50-ml Falcon tubes or Whirl-Pak bags and placed on ice until preservation. For preservation, residual seawater was decanted and replaced with RNA/DNA stabilizing buffer, and the sample was then frozen. To sample the coral microbiome, small (~1 cm²) fragments of massive *Porites* sp. coral (including mucus, tissue, and skeleton) were removed from the coral head by use of a hammer and chisel. Coral fragments were placed in a Whirl-Pak bag underwater and immediately brought to the surface, where the seawater was decanted and replaced with RNA/DNA stabilizing buffer. Samples were frozen upon return to the research station. Samples of algae were picked by hand and placed in 0.2-μm-filtered seawater in sterile 50-ml Falcon tubes, which were then stored on ice. The tubes containing the algae were vortexed for 5 min, and the water was then filtered through a 0.2-μm Isopore membrane filter (Millipore) to capture suspended microbes. Both the filter and a small algal fragment from the same sample were immersed in RNA/DNA stabilizing buffer and frozen.

This research was approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee (IACUC) under protocol A15094.

DNA extraction. Total DNA was extracted from all samples by use of MoBio PowerSoil DNA extraction kits. Cryovials containing the preserved samples were thawed on ice. For gills and sediment, subsamples (≤1 cm² gill fragment; ~0.25 g sediment) were placed directly into PowerBead tubes, and extraction was performed following the manufacturer's instructions. For intestinal samples, either the entire intestine (recruits) or fragments of the intestine (adults) were placed directly into a PowerBead tube. The tube was vortexed for 15 s to dispel intestinal content, the intestinal lining was subsequently removed, and extraction was performed following the manufacturer's instructions. For alga, coral, and seawater samples, the entire original sample (filter plus algal fragment, coral fragment, and filter, respectively) was placed directly into a PowerBead tube, and extraction was performed following the manufacturer's instructions.

Host identification and diet type determination. Where possible, host taxonomic identification was determined by comparing specimens to a standard reference book (48). For each identified taxon, the nucleotide sequence of the cytochrome *c* oxidase 1 (COI) gene was obtained from Fishbase (www.fishbase.org). Any fish that could not be identified was genotyped according to previously described methods (49). Briefly, the COI gene was amplified by PCR, using primers C_FishF1t1 and C_FishR1t1 in 25-μl reaction mixtures with gill DNA as the template (Table 5). PCR products were verified in a 0.8% agarose gel, visualized with GelRed (Biotium), and purified using an ExSap-IT PCR product cleanup kit (Affymetrix). Cleaned PCR products were then sequenced bidirectionally using Sanger technology. All generated and Fishbase-obtained sequences were then aligned using ClustalW (50) and used to estimate the host phylogeny by use of maximum likelihood (ML) with the Kimura two-parameter model of substitution in MEGA (51), with clade support assessed via bootstrapping (1,000 replicates). The ML topology was visualized using the Interactive Tree of Life (52). The broad diet category (omnivore, herbivore, or carnivore) for each host species was estimated based on a comprehensive literature review, the results of which are shown in Table S1.

Microbiome data analysis. Microbial community composition was assessed by Illumina sequencing of the V3-V4 region of the 16S rRNA gene. The V3-V4 region was PCR amplified using primers F515 and R806 (53), each appended with barcodes and Illumina-specific adapters as described previously (54) (Table 5). Reaction mixtures included 1 to 2 μl DNA template, 22 μl Platinum PCR SuperMix (Life Technologies), 0.5 μl (each) forward and reverse primers (total concentration, 0.4 μM), and 1 to 2 μl bovine serum albumin (BSA) (20 mg/ml; New England BioLabs Inc.). PCR conditions were an initial 3-min denaturation at 94°C, followed by 30 cycles of denaturation at 94°C (45 s), primer annealing at 55°C (45 s), and primer extension at 72°C (90 s) and then a final extension at 72°C for 10 min. Amplicon libraries were purified by use of Diffnity RapidTip PCR purification tips (Diffnity Genomics, NY), quantified on a Qubit machine (Life Technologies), and pooled at equimolar concentrations. Amplicons were sequenced on an Illumina MiSeq machine across six different runs, using a V2 500-cycle kit (250 × 250 bp) with 10% PhiX to increase read diversity.

Sequences were trimmed using Trim Galore, with the criteria of a minimum length of 100 bp and a Phred score of >25. Paired reads were then merged using FLASH (55), with the criteria of a minimum length of 250 bp per input read, a minimum length of 300 bp for merged fragments, and a maximum

fragment standard deviation of 30 bp. Merged reads were imported into QIIME, and chimeric sequences were detected by searches using uchime_ref in USEARCH against the Greengenes database (56) and then removed. Sequences sharing 97% nucleotide similarity were clustered into operational taxonomic units (OTUs) by open-reference picking with the uclust tool (57), with taxonomy assigned to OTUs by comparison to the Greengenes database (56). Singletons were excluded from the analysis; all OTUs reported here are represented by at least two sequences. Sequences classified as “chloroplast” or not classified as “bacteria” or “archaea” were removed. QIIME OTU tables were used for further analyses as described below.

Statistical analyses. We assessed alpha and beta diversity values by using rarefied OTU data based on a uniform depth of 3,000 sequences (sequence counts per sample ranged from 3,073 to 234,562). Chao1 estimates of OTU richness (alpha diversity) were obtained through QIIME. Bray-Curtis dissimilarity matrices were calculated based on the rarefied OTU data and used to measure beta diversity as the distance from individual samples to the centroid of the sample type (i.e., dispersion), using PERMANOVA in the program PRIMER 7 (Primer-E Ltd.). We tested for significant variation in both alpha (Chao1) and beta (dispersion) diversity among samples by using analysis of variance (ANOVA) followed by Tukey *post hoc* tests. The Bray-Curtis matrices were also used for nonmetric multidimensional scaling (NMDS) and an analysis of similarity (ANOSIM) to test for variation in community composition among sample groups, also in PRIMER 7. The latter involved a one-way ANOSIM using life stage (recruit and adult) and sample type (gill and intestine) as factors. Four separate one-way analyses were conducted for each sample type (recruit or adult $\times$ gill or intestine), using presumed diet category (herbivore, carnivore, or omnivore) (Table S1) as a factor.

To assess the potential of host-specific factors to influence microbiome structuring across body sites, we tested whether similarity between gill and intestinal microbiomes varies within versus between individuals. For this analysis, we used data only for individuals for which we had paired gill and intestine data sets ($n = 24$ for recruits; $n = 110$ for adults), using a uniform number of sequences ($n = 3,000$) per data set. The Bray-Curtis similarity between gill and intestinal microbiomes (evaluated at the OTU level) was calculated for each individual and then averaged across individuals (within individual). Mean within-individual similarity was compared by use of the *t* test (two-tailed) to between-individual similarity, obtained by calculating the mean Bray-Curtis similarity for all possible pairwise gill versus intestine comparisons involving data sets from different individuals. We also conducted this analysis after partitioning samples based on host taxon, focusing on the following three fish families represented by multiple individuals in both adult and recruit sample sets: Chaetodontidae, Pomacanthidae, and Holocentridae.

Nonrarefied count data were used to detect OTUs that differed significantly in proportional abundance among sample groups. For this analysis, the OTU table containing absolute sequence counts was reformatted in Phyloseq (58), and the program DESeq2 (59) was used to detect differentially abundant taxa. Nonrarefied data were used because the use of rarefied data for differential abundance testing has been shown to lead to false-negative results (lower power) due to the exclusion of data (60). DESeq2 performs an internal variance stabilization procedure that adjusts nonrarefied counts via a log-like transformation to account for variation in sequencing depth.

Accession number(s). All sequence data generated in this study have been deposited in the NCBI Sequence Read Archive under BioProject ID numbers [PRJNA401167](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA401167) and [PRJNA401168](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA401168).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00063-18>.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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Z.A.P. and F.J.S. designed the study. Z.A.P. and M.B. designed the sampling strategy and collected samples. Z.A.P. and R.D.H. processed samples. Z.A.P. and F.J.S. analyzed and interpreted data and prepared the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Lieske E, Myers R. 2002. Coral reef fishes: Indo-Pacific and Caribbean. Princeton University Press, Princeton, NJ.
2. Parris DJ, Brooker RM, Morgan MA, Dixon DL, Stewart FJ. 2016. Whole gut microbiome composition of damselfish and cardinalfish before and after reef settlement. *PeerJ* 4:e2412. <https://doi.org/10.7717/peerj.2412>.
3. Ngugi DK, Miyake S, Cahill M, Vinu M, Hackmann TJ, Blom J, Tietbohl MD,

- Berumen ML, Stingl U. 2017. Genomic diversification of giant enteric symbionts reflects host dietary lifestyles. *Proc Natl Acad Sci U S A* 114:E7592–E7601. <https://doi.org/10.1073/pnas.1703070114>.
4. Lescak EA, Milligan-Myhre KC. 2017. Teleosts as model organisms to understand host-microbe interactions. *J Bacteriol* 199:e00868–16. <https://doi.org/10.1128/JB.00868-16>.
 5. Maina JN. 2002. Structure, function and evolution of the gas exchangers: comparative perspectives. *J Anat* 201:281–304. <https://doi.org/10.1046/j.1469-7580.2002.00099.x>.
 6. Cantley AM, Clardy J. 2015. Animals in a bacterial world: opportunities for chemical ecology. *Nat Prod Rep* 32:888–892. <https://doi.org/10.1039/C4NP00141A>.
 7. Gilbert JA, Quinn RA, Debelius J, Xu ZZ, Morton J, Garg N, Jansson JK, Dorrestein PC, Knight R. 2016. Microbiome-wide association studies link dynamic microbial consortia to disease. *Nature* 535:94–103. <https://doi.org/10.1038/nature18850>.
 8. Chemical Sciences Roundtable, Board on Chemical Sciences and Technology, Division on Earth and Life Studies, National Academies of Sciences, Engineering, and Medicine. 2017. The chemistry of microbiomes: proceedings of a seminar series. The National Academies Press, Washington, DC.
 9. Lee W-J, Hase K. 2014. Gut microbiota-generated metabolites in animal health and disease. *Nat Chem Biol* 10:416. <https://doi.org/10.1038/nchembio.1535>.
 10. Wilkie MP. 2002. Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. *J Exp Zool* 293: 284–301. <https://doi.org/10.1002/jez.10123>.
 11. Hess S, Wenger AS, Ainsworth TD, Rummer JL. 2015. Exposure of clownfish larvae to suspended sediment levels found on the Great Barrier Reef: impacts on gill structure and microbiome. *Sci Rep* 5:10561. <https://doi.org/10.1038/srep10561>.
 12. Llewellyn MS, Boutin S, Hoseinifar SH, Derome N. 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Front Microbiol* 5:207. <https://doi.org/10.3389/fmicb.2014.00207>.
 13. Lowrey L, Woodhams DC, Tacchi L, Salinas I. 2015. Topographical mapping of the rainbow trout (*Oncorhynchus mykiss*) microbiome reveals a diverse bacterial community with antifungal properties in the skin. *Appl Environ Microbiol* 81:6915–6925. <https://doi.org/10.1128/AEM.01826-15>.
 14. van Kessel MAHJ, Mesman RJ, Arshad A, Metz JR, Spanings TFA, van Dalen SCM, van Niftrik L, Flik G, Wendelaar Bonga SE, Jetten MS, Klaren PH, Op den Camp HJ. 2016. Branchial nitrogen cycle symbionts can remove ammonia in fish gills. *Environ Microbiol Rep* 8:590–594. <https://doi.org/10.1111/1758-2229.12407>.
 15. Schram E, Roques JAC, Abbink W, Spanings T, de Vries P, Bierman S, van de Vis H, Flik G. 2010. The impact of elevated water ammonia concentration on physiology, growth and feed intake of African catfish (*Clarias gariepinus*). *Aquaculture* 306:108–115. <https://doi.org/10.1016/j.aquaculture.2010.06.005>.
 16. Reverter M, Sasal P, Tapissier-Bontemps N, Lecchini D, Suzuki M. 2017. Characterisation of the gill mucosal bacterial communities of four butterflyfish species: a reservoir of bacterial diversity in coral reef ecosystems. *FEMS Microbiol Ecol* 93:6. <https://doi.org/10.1093/femsec/fix051>.
 17. Hennersdorf P, Kleinert S, Theisen S, Abdul-Aziz MA, Mrotzek G, Palm HW, Saluz HP. 2016. Microbial diversity and parasitic load in tropical fish of different environmental conditions. *PLoS One* 11:e0151594. <https://doi.org/10.1371/journal.pone.0151594>.
 18. Givens C, Ransom B, Bano N, Hollibaugh T. 2015. Comparison of the gut microbiomes of 12 bony fish and 3 shark species. *Mar Ecol Prog Ser* 518:209–223. <https://doi.org/10.3354/meps11034>.
 19. Alcaraz LD, Hernández AM, Peimbert M. 2016. Exploring the cockatiel (*Nymphicus hollandicus*) fecal microbiome, bacterial inhabitants of a worldwide pet. *PeerJ* 4:e2837. <https://doi.org/10.7717/peerj.2837>.
 20. Thompson LR, Sanders JG, McDonald D, Amir A, Ladau J, Locay KJ, Prill RJ, Tripathi A, Gibbons SM, Ackermann G, Navas-Molina JA, Janssen S, Kopylova E, Vázquez-Baeza Y, González A, Morton JT, Mirarab S, Zech X, Jiang L, Haroon MF, Kanbar J, Zhu Q, Jin Song S, Kosciolk T, Bokulich NA, Lefler J, Brislawn CJ, Humphrey G, Owens SM, Hampton-Marcell J, Berg-lyons D, McKenzie V, Fierer N, Fuhrman JA, Clauser A, Stevens RL, Shade A, Pollard KS, Goodwin KD, Jansson JK, Gilbert JA, Knight R, Earth Microbiome Project Consortium. 2017. A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* 551:457–463. <https://doi.org/10.1038/551033a>.
 21. Saeed MO, Alamoudi MM, Al-Harbi AH. 1990. Histopathology of *Pseudomonas putrefaciens* associated with disease in cultured rabbitfish, *Siganus rivulatus* (Forsk.). *J Fish Dis* 13:417–422. <https://doi.org/10.1111/j.1365-2761.1990.tb00801.x>.
 22. Liu H, Guo X, Gooneratne R, Lai R, Zeng C, Zhan F, Wang W. 2016. The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. *Sci Rep* 6:24340. <https://doi.org/10.1038/srep24340>.
 23. Neave MJ, Rachmawati R, Xun L, Michell CT, Bourne DG, Apprill A, Voolstra CR. 2017. Differential specificity between closely related corals and abundant *Endozoicomonas* endosymbionts across global scales. *ISME J* 11:186–200. <https://doi.org/10.1038/ismej.2016.95>.
 24. Pratte ZA, Longo GO, Burns AS, Hay ME, Stewart FJ. 2018. Contact with turf algae alters the coral microbiome: contact versus systemic impacts. *Coral Reefs* 37:1–13. <https://doi.org/10.1007/s00338-017-1615-4>.
 25. Ding J-Y, Shiu J-H, Chen W-M, Chiang Y-R, Tang S-L. 2016. Genomic insight into the host-endosymbiont relationship of *Endozoicomonas montiporae* CL-33(T) with its coral host. *Front Microbiol* 7:251. <https://doi.org/10.3389/fmicb.2016.00251>.
 26. Mendoza M, Güiza L, Martínez X, Caraballo X, Rojas J, Aranguren LF, Salazar M. 2013. A novel agent (*Endozoicomonas elysicola*) responsible for epitheliocystis in cobia *Rachycentrum canadum* larvae. *Dis Aquat Organ* 106:31–37. <https://doi.org/10.3354/dao02636>.
 27. Katharios P, Seth-Smith HMB, Fehr A, Mateos JM, Qi W, Richter D, Nufer L, Ruettgen M, Guevara Soto M, Ziegler U, Thomson NR, Schlapbach R, Vaughan L. 2015. Environmental marine pathogen isolation using mesocosm culture of sharpnose seabream: striking genomic and morphological features of novel *Endozoicomonas* sp. *Sci Rep* 5:17609. <https://doi.org/10.1038/srep17609>.
 28. Larsen AM, Bullard SA, Womble M, Arias CR. 2015. Community structure of skin microbiome of gulf killifish, *Fundulus grandis*, is driven by seasonality and not exposure to oiled sediments in a Louisiana salt marsh. *Microb Ecol* 70:534–544. <https://doi.org/10.1007/s00248-015-0578-7>.
 29. Carlson JM, Leonard AB, Hyde ER, Petrosino JF, Primm TP. 2017. Microbiome disruption and recovery in the fish *Gambusia affinis* following exposure to broad-spectrum antibiotic. *Infect Drug Resist* 10:143–154. <https://doi.org/10.2147/IDR.S129055>.
 30. Larsen A, Tao Z, Bullard SA, Arias CR. 2013. Diversity of the skin microbiota of fishes: evidence for host species specificity. *FEMS Microbiol Ecol* 85:483–494. <https://doi.org/10.1111/1574-6941.12136>.
 31. Ditttrich S, Phuklia W, Turner GDH, Rattanavong S, Chansamouth V, Dumler SJ, Ferguson DJ, Paris DH, Newton PN. 2015. *Neorickettsia senetsu* as a neglected cause of fever in South-East Asia. *PLoS Negl Trop Dis* 9:e0003908. <https://doi.org/10.1371/journal.pntd.0003908>.
 32. Boutin S, Sauvage C, Bernatchez L, Audet C, Derome N. 2014. Inter individual variations of the fish skin microbiota: host genetics basis of mutualism? *PLoS One* 9:e102649. <https://doi.org/10.1371/journal.pone.0102649>.
 33. Wilson B, Danilowicz BS, Meijer WG. 2008. The diversity of bacterial communities associated with Atlantic cod *Gadus morhua*. *Microb Ecol* 55:425–434. <https://doi.org/10.1007/s00248-007-9288-0>.
 34. Smith CJ, Danilowicz BS, Meijer WG. 2009. Bacteria associated with the mucus layer of *Merlangius merlangus* (whiting) as biological tags to determine harvest location. *Can J Fish Aquat Sci* 66:713–716. <https://doi.org/10.1139/F09-035>.
 35. Salinas I. 2015. The mucosal immune system of teleost fish. *Biology* 4:525–539. <https://doi.org/10.3390/biology4030525>.
 36. Zhang Y-A, Salinas I, Li J, Parra D, Bjork S, Xu Z, LaPatra SE, Bartholomew J, Sunyer JO. 2010. IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat Immunol* 11:827–835. <https://doi.org/10.1038/ni.1913>.
 37. Ip YK, Chew SF. 2010. Ammonia production, excretion, toxicity, and defense in fish: a review. *Front Physiol* 1:134. <https://doi.org/10.3389/fphys.2010.00134>.
 38. Tarnecki AM, Patterson WF, Arias CR. 2016. Microbiota of wild-caught red snapper *Lutjanus campechanus*. *BMC Microbiol* 16:245. <https://doi.org/10.1186/s12866-016-0864-7>.
 39. Shephard KL. 1994. Functions for fish mucus. *Rev Fish Biol Fish* 4:401–429. <https://doi.org/10.1007/BF00042888>.
 40. Subramanian S, Ross NW, MacKinnon SL. 2008. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. *Comp Biochem Physiol B Biochem Mol Biol* 150:85–92. <https://doi.org/10.1016/j.cbpb.2008.01.011>.
 41. Xu Z, Parra D, Gomez D, Salinas I, Zhang Y-A, von Gersdorff Jorgensen

- L, Heinecke RD, Buchmann K, LaPatra S, Sunyer JO. 2013. Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proc Natl Acad Sci U S A* 110:13097–13102. <https://doi.org/10.1073/pnas.1304319110>.
42. Xu Z, Takizawa F, Parra D, Gómez D, von Gersdorff Jørgensen L, LaPatra SE, Sunyer JO. 2016. Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods. *Nat Commun* 7:10728. <https://doi.org/10.1038/ncomms10728>.
 43. Schmidt V, Amaral-Zettler L, Davidson J, Summerfelt S, Good C. 2016. Influence of fishmeal-free diets on microbial communities in Atlantic salmon (*Salmo salar*) recirculation aquaculture systems. *Appl Environ Microbiol* 82:4470–4481. <https://doi.org/10.1128/AEM.00902-16>.
 44. Burns AR, Stephens WZ, Stagaman K, Wong S, Rawls JF, Guillemin K, Bohannon BJ. 2016. Contribution of neutral processes to the assembly of gut microbial communities in the zebrafish over host development. *ISME J* 10:655–664. <https://doi.org/10.1038/ismej.2015.142>.
 45. Holzer G, Besson M, Lambert A, François L, Barth P, Gillet B, Hughes S, Piganeau G, Leulier F, Viriot L, Lecchini D, Laudet V. 2017. Fish larval recruitment to reefs is a thyroid hormone-mediated metamorphosis sensitive to the pesticide chlorpyrifos. *Elife* 6:e27595. <https://doi.org/10.7554/eLife.27595>.
 46. Besson M, Salis P, Laudet V, Lecchini D. 2018. Complete and rapid reversal of the body color pattern in juveniles of the convict surgeonfish *Acanthurus triostegus* at Moorea Island (French Polynesia). *Coral Reefs* 37:31–35. <https://doi.org/10.1007/s00338-017-1631-4>.
 47. Dufour V, Galzin R. 1993. Colonization patterns of reef fish larvae to the lagoon at Moorea Island, French Polynesia. *Mar Ecol Prog Ser* 102:143–152. <https://doi.org/10.3354/meps102143>.
 48. Allen GR, Erdmann M. 2012. Reef fishes of the East Indies: Andaman Sea, Myanmar, Thailand, Indonesia, Christmas Island, Singapore, Malaysia, Brunei, Philippines, Papua New Guinea, Solomon Islands. Tropical Reef Research, Singapore.
 49. Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN. 2007. Universal primer cocktails for fish DNA barcoding. *Mol Ecol* 7:544–548. <https://doi.org/10.1111/j.1471-8286.2007.01748.x>.
 50. Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680. <https://doi.org/10.1093/nar/22.22.4673>.
 51. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33:1870–1874. <https://doi.org/10.1093/molbev/msw054>.
 52. Letunic I, Bork P. 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res* 44:242–245. <https://doi.org/10.1093/nar/gkw290>.
 53. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* 108(Suppl 1):4516–4522. <https://doi.org/10.1073/pnas.1000080107>.
 54. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 79:5112–5120. <https://doi.org/10.1128/AEM.01043-13>.
 55. Magoč T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27:2957–2963. <https://doi.org/10.1093/bioinformatics/btr507>.
 56. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 72:5069–5072. <https://doi.org/10.1128/AEM.03006-05>.
 57. Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461. <https://doi.org/10.1093/bioinformatics/btq461>.
 58. McMurdie PJ, Holmes S. 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. <https://doi.org/10.1371/journal.pone.0061217>.
 59. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15:550. <https://doi.org/10.1186/s13059-014-0550-8>.
 60. Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y, Birmingham A, Hyde ER, Knight R. 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* 5:27. <https://doi.org/10.1186/s40168-017-0237-y>.