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Characterization of the microbiome and bioluminescent symbionts across life stages of ceratioid anglerfishes of the gulf of mexico

Lindsay L. Freed
Nova Southeastern University, lf712@mynsu.nova.edu

Cole Easson
Nova Southeastern University, ceasson@nova.edu

Lydia Baker
Cornell University

D. Fenolio
San Antonio Zoo, dantefenolio@sazoo.org

Tracey Sutton
Nova Southeastern University, tsutton1@nova.edu

See next page for additional authors

Authors
Lindsay L. Freed, Cole Easson, Lydia Baker, D. Fenolio, Tracey Sutton, Yasmin Khan, Patricia Blackwelder, Tory Hendry, and Jose Lopez

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Authors:

Lindsay L. Freed [a], Cole Easson[a], Lydia J. Baker[b], Danté Fenolio[c], Tracey T. Sutton[a] , Yasmin Khan, Patricia Blackwelder[a,d], Tory A. Hendry[b], Jose V. Lopez[a]

[a]Halmos College of Natural Sciences and Oceanography, Nova Southeastern University, Dania Beach, Florida, USA

[b]Department of Microbiology, Cornell University, Ithaca, New York, USA

[c]Center for Conservation and Research, San Antonio Zoo, San Antonio, Texas, USA

[d]University of Miami Center for Advanced Microscopy, Department of Chemistry, University of Miami, Coral Gables, Florida, USA

Corresponding Author: Jose Lopez joslo@nova.edu

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ABSTRACT - 200

The interdependence of diverse organisms through symbiosis reaches even the deepest parts of the oceans. As part of the DEEPEND project (deependconsortium.org) research on deep Gulf of Mexico biodiversity, we profiled the bacterial communities ("microbiomes") and luminous symbionts of 36 specimens of adult and larval deep-sea anglerfishes of the suborder Ceratioidei using 16S rDNA. Transmission Electron Microscopy was used to characterize the location of symbionts in adult light organs (esca). Whole larval microbiomes, and adult skin and gut microbiomes, were dominated by bacteria in the genera *Moritella* and *Pseudoalteromonas* genera. 16S rDNA sequencing results from adult fishes corroborate the previously published identity of ceratioid bioluminescent symbionts and support findings that these symbionts do not consistently exhibit host specificity at the host family level. Bioluminescent symbiont amplicon sequence variants (ASVs) were absent from larval ceratioid samples, but were found at all depths in the seawater, with a highest abundance found at mesopelagic depths. As adults spend the majority of their lives in the meso and bathypelagic, the trend in symbiont abundance is consistent with their life history. These findings support the hypothesis that bioluminescent symbionts are not present throughout host development, and that ceratioids acquire their bioluminescent symbionts from the environment.

INTRODUCTION

Bioluminescent symbiosis has evolved repeatedly in diverse lineages of marine fishes, such as those that live in one of the largest habitats of the planet, deep-sea anglerfishes (Herring & Morin, 1978; Dunlap & Urbanczyk, 2013; Dunlap et al, 2014; Davis et al., 2016). Anglerfishes, inhabiting the disphotic/aphotic zones of the oceans below 200

m, have been collected in every major ocean across the world (Pietsch 2009). These fishes comprise the suborder Ceratioidei, the most speciose vertebrate taxon in the in the bathypelagic zone (oceanic waters deeper than 1,000 m) (Pietsch 2009; Miya et al., 2010). Female deep-sea anglerfishes belonging to nine families develop a lure (esca) containing bioluminescent bacterial symbionts (Leisman et al. 1980). Although ceratioid anglerfishes are common in the deep sea, they are difficult to collect, historically making studies of their associated microbes and symbionts challenging.

As in other marine animals, anglerfishes are constantly exposed to environmental bacteria. Previous studies have used comparisons of host and environmental microbiomes to infer bacterial communities that are host-associated (Larsen *et al.* 2015; Legrand *et al.* 2018; Pratte *et al.* 2018). There are various morphological and environmental factors that may impact the microbiome of anglerfishes. Anglerfish skin is relatively mucose from polysaccharides and mostly scaleless, which may provide a potential carbon source to piezophilic (high pressure adjusted) bacteria in the nutrient-limited deep sea (Hansell 2013, Pietsch 2009). Anglerfish are also covered by sensory papillae (Pietsch 2009), which provides increased surface area and possible microenvironments for bacterial colonization. The potentially unique microbiome of deep-sea anglerfishes have not yet been characterized, but as in other animals, it is likely to impact the health and phenotypes of these fishes.

In addition to interacting with their microbiome, female anglerfish also establish a symbiosis with bioluminescent bacteria (Haygood *et al.*, 1992; Haygood and Distel, 1993). Luminous anglerfishes host symbiotic bacteria in the esca, a specialized organ that tops a modified dorsal ray (illicium) (Munk 1999). In the most basic sense, the esca is a spherical, bacteria-filled organ that contains one or more small openings to the external environment.

However, there is more structural complexity as these organs can also contain lenses, filters, reflectors, filaments, and multiple appendages (Munk 1999). It is thought that anglerfishes are capable of controlling the bacterial populations within the esca by altering the conditions within the organ (Pietsch 2009). These bioluminescent lures may be used for mate-finding purposes in addition to prey attraction (Herring 2000, 2007). In addition to the esca, females within the family Ceratiidae possess a modified anterior dorsal-fin ray, called a caruncle, which is similar in form to the esca. Members of the genus *Ceratias* develop two caruncles, while members of the genus *Cryptopsaras* develop three. Histological study and genome sequence analyses of *Cryptopsaras couesii* caruncle symbionts, like the esca, contain dense populations of luminous bacteria that can be expelled through a distal pore (Hansen and Herring 1977; Herring and Morin 1978, Baker *et al.*, *in review*).

Since the bacteria contained within anglerfish escae have historically proven to be unculturable via traditional laboratory techniques (Haygood and Distel, 1993), genomic methods have only recently confirmed the identity of these symbionts. Hendry et al. (2018) characterized the genomes of bioluminescent symbionts from two *Cryptopsaras couseii* and a single *Melanocetus johnsonii* and found that they hosted novel bacterial species named "*Candidatus* Enterovibrio luxaltus" and "*Candidatus* Enterovibrio escacola" respectively. This study also suggested that ceratioid symbionts are potentially engaged in an obligate relationship with their hosts, due to extreme genome reduction and loss of metabolic abilities in the bacterial genomes. Although these patterns are common to vertically transmitted symbionts (Bright and Bulgheresi, 2010; McCutcheon and Moran, 2011), recent work found support for the hypothesis that anglerfish acquire their symbionts from environmental populations (Baker *et al.*, *in review*). This style of environmental transmission is also found in most other symbiotically luminous fishes (Dunlap *et al.* 2007;

Dunlap and Urbanczyk, 2013; Urbanczyk et al, 2010) and other deep-sea organisms (Rouse *et al*, 2009).

However, larval anglerfishes do not possess a lure capable of housing the symbiotic bacteria (Munk and Herring 1996). It is not until the larvae metamorphose that the juveniles perform a vertical migration to the mesopelagic and deeper. During development, the primordial esca invaginates to create a cavity capable of holding bacteria (Munk, *et al*., 2009; Pietsch 2009). It has also been proposed that the female anglerfish may inoculate her eggs with the symbiont before the absorbent and buoyant egg raft makes its way towards the ocean surface where the larvae will hatch (Pietsch, 2009; Fukui *et al.*, 2010; Dunlap *et al.*, 2014). Alternatively, symbiosis may initiate during anglerfish adulthood as bacteria can enter the escal pore, as it is continuously exposed to the external environment (Munk and Herring, 1996; Munk 1999). It has not yet been well established whether the ceratioid symbionts identified by Hendry et al. (2018) are present in the environment and absent in larval anglerfishes, which would help confirm the hypothesis that anglerfishes acquire their symbionts from environmental populations (Baker *et al.*, *in review*).

In this study we aim to characterize the microbial communities found on both adult and larval anglerfishes. This data improves our understanding of the bacterial populations associated with these speciose, but difficult to study, fishes and was also used to define the distribution of luminous anglerfish symbionts across diverse fish species and within larvae. In addition to attempting to detect symbionts in larvae, seawater samples from the Gulf of Mexico were examined for the presence of symbiont taxa to confirm the likelihood of anglerfish bioluminescent symbionts being acquired from the environment rather than vertically transmitted between host generations.

MATERIALS & METHODS

Sample Collection and Processing

All anglerfish and seawater samples were collected over the course of four DEEPEND cruises aboard the *R/V Point Sur* in the Gulf of Mexico: DP01 from May 1 – 8, 2015, DP02 from August 8-21, 2015, DP03 from April 20 – May 14, 2016, and DP04 from August 5-19, 2016. Previously established SEAMAP station locations were used for labeling collection sites (www.gsmfc.org). All anglerfish specimens were collected using a 10 m^2 mouth area, six-net MOCNESS (Multiple Opening and Closing Environmental Sensing System) with 3-mm mesh (Wiebe *et al.* 1976) with nets deployed at the following targeted depths: Net0 0-1500 m, Net1 1500-1200 m, Net2 1200-1000 m, Net3 1000-600 m, Net4 600-200 m, Net5 200-0 m.

Water samples were collected at each station using CTD Niskin bottle rosette. During each cast, water samples were collected at up to five depths. Four to five liters of seawater were collected from each sampled depth and separated into three one-liter replicates that were then filtered through a 0.45-micron filter (Daigger) under low pressure using a vacuum pump (Easson and Lopez, 2019). All water specimens were stored at -80°C until processing by the Microbiology & Genetics Laboratory at Nova Southeastern University Halmos College of Natural Sciences and Oceanography. Reports for each of the four cruises can be found as supplemental data or at the DEEPENDconsortium.org website.

Specimen Taxonomy

Once onboard, anglerfish specimens were sorted, identified to the lowest taxonomic level possible, and placed in ethanol or RNAlater by DEEPEND Consortium's Chief Scientist Tracey Sutton (Sutton *et al.* 2010; Pietsch and Sutton 2015).

Transmission Electron Microscopy Methods

The esca from one *C. couesii* was dissected and placed in a fixative solution of 2% gluteraldehyde in 0.05M sodium cacodylate made with pre-filtered (0.22-micron filter) seawater. The esca was then washed with fresh fixative overnight. The structure was then rinsed in three changes of the buffer solution and post-fixed in 1% osmium tetroxide in buffer for 1 hour, rinsed in three changes of buffer, and dehydrated in a graded series of ethanol to 100%. The esca was then embedded in Spurr embedding resin and sectioned with a Porter Blum Ultramicrotome. The sections were then mounted on copper grids, coated with formvar and carbon and examined using a JEOL 1400X transmission electron microscope located at the TEM Core in the Miller School of Medicine at the University of Miami. Micrographs were taken using a Gatan digital camera.

Microbial DNA Extraction

Anglerfish specimens were dissected with sterilized instruments. For specimens collected during cruises DP01 and DP02, the entire luring apparatus (esca and illicium) were dissected and preserved while at sea as a single sample labeled “esca”. During later cruises (DP03 and DP04), the entire lure apparatus was dissected and preserved while at sea, but prior to DNA extract at NSU, it was further dissected into two separate specimens labeled as the esca and illicium accordingly. For ceratiid specimens, the base of the caruncles was separated from the back of the fish and all two or three caruncles, depending on anglerfish species, were included in the sample. The least damaged pectoral fin was dissected as well as an undamaged portion of skin from the lateral side of the anglerfishes. For gill sample dissection, the gill-filaments, gill-rakers, and gill arch were removed from one side of the anglerfish. Lastly, the entire intestine, from the base of the stomach to the cloaca was extracted for the gut sample.

All microbial DNA isolations were conducted following the Earth Microbiome Project (earthmicrobiome.org) protocol with the MO BIO PowerLyzer™ PowerSoil® kit. After extraction a 1% agarose gel was run to ensure that the DNA extraction was successful. After gel verification the DNA concentration was confirmed using a Qubit 2.0 (Life Technologies) fluorometer.

Illumina High-Throughput Metagenomic Sequencing

All samples were prepared for sequencing following the 16S Illumina Amplicon Protocol per the Earth Microbiome Project (Caporaso *et al.* 2011). The 806R and 515F primers were used for PCR amplification of the V4 region of the 16S rRNA gene (Caporaso *et al.* 2011). Amplicons were sequenced with an Illumina MiSeq using the V2 500-cycle cartridge across three runs to generate paired-end 250 base pair amplicons (Caporaso *et al.* 2012). Quality filtering was performed using Trimmomatic 0.36 to remove the following: adapters (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10); low-quality leading and trailing bases (LEADING: 3, TRAILING 3); reads with an average base quality below 15 (SLIDINGWINDOW:4:15); reads below 24 bases long (MINLEN:24). Further analysis was limited to paired-end reads that passed quality filtering.

Sequencing Analysis using QIIME

The initial processing of raw microbiome data was performed using Quantitative Insights into Microbial Ecology, QIIME2 (qiime2, version 2018.11, https//doc.qiime2.org/2018.11) (Caporaso *et al.* 2010). The plugins used were demux (https://github.com/qiime2/q2-demux), dada2 (Callahan et al., 2016) and feature-table. The amplicon sequence variants (ASVs) were assigned using feature-classifier (https://github.com/qiime2/q2-feature-classifier) and taxa (https://github.com/qiime2/q2-taxa) at 99% using the Silva classifier 132-99-515-806 (Quast 2013).

Community Analysis using R

Analysis was executed with the RStudio software (version 3.5.1)(R Core Team 2016), with the added packages 'phyloseq' and 'vegan' which were developed to examine microbial ecology (McMurdie and Holmes 2013; Oksanen *et al.* 2018). Seawater replicates were merged into a single sample per collection depth and location. All samples were then rarefied to a uniform depth of 1000 sequences and were transformed to reflect relative abundance. Variations associated with sample type (anglerfish or water), organ type (esca, caruncle, illicium, fin, gill, gut, or skin), and anglerfish developmental stage (larval, post-larval, or adult) were analyzed using 'phyloseq' and 'vegan'.

Alpha diversity was measured by calculating ASV observed richness, Chao1 index, Shannon index, and the Inverse Simpson's index for each sample type, anglerfish organ type, and anglerfish developmental stage using phyloseq (McMurdie and Holmes 2013). Differences in alpha diversity among sample type, organ type, and developmental stage were assessed using an analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) post hoc test to determine pairwise differences.

Beta diversity was estimated by calculating Bray-Curtis dissimilarity to determine differences in the community composition by sample type, anglerfish organ type, and anglerfish developmental stage. Dissimilarity was presented as distance matrices and a permuted multivariate ANOVA (Adonis) was used to assess significant differences. Lastly, a SIMPER test with 499 permutations was used to show which specific taxa were driving differences between sample type and organ type microbiomes.

Symbiont Analysis using R

For symbiont analysis, the original, unrarefied dataset was used so as not to exclude rare taxa that may have been inadvertently excluded when normalizing to a uniform depth

of 1000 sequences. For this dataset, 16S rRNA sequence data were transformed to reflect relative abundance. To identity all potential anglerfish symbiont taxa, even any that have not been previously described, only Vibrionaceae ASVs (relative abundance >0.1%), as Vibrionaceae contains known bioluminescent symbionts of fishes (Dunlap and Urbanczyk 2013). These were examined on all tissue samples of adult anglerfish samples. These potential symbiont ASV sequences were evaluated further using the BLAST database (Altschul et al., 1997) as well as direct BLAST to the metagenomic sequencing results of the previously documented symbionts, *E. luxaltus* and *E. escacola* (Hendry et al., 2018, Baker *et al*., in review). Sequences that made up greater than 10% relative abundance are refered to as confirmed symbionts. Larval anglerfish samples, which had a lower abundance of Vibrionaceae ASVs, were examined for symbiont ASVs that were previously identified as adult samples, as were other anglerfish organ types and within water samples.

RESULTS

Anglerfish specimen collection

A total of 36 anglerfish specimens were collected over the course of four DEEPEND cruises. Based on morphology, specimens from six families were identified: Ceratiidae (n=22), Oneirodidae (n=7), Linophrynidae (n=3), Melanocetidae (n=2), Centrophrynidae (n=1), and Gigantactinidae (n=1). Further resolution was determined to a minimum of eight anglerfish species shown in Table 1, and a subset of these samples were also confirmed using mitochondrial DNA (Baker et al., *in review*). More accurate taxonomic resolution was difficult because larval anglerfish specimens were sometimes involved. However, characterizations are continuing in other studies of the DEEPEND consortium and beyond the scope of this paper.

Transmission electron microscopy of esca

To show physical evidence of anglerfish esca-specific symbionts, transmission electron microscopy was performed on a representative esca of one sequenced *Cryptopsaras couesii* sample (CC70, DP04-12AUG16-MOC10-SW5D-070-N3) (Fig. 1A). The image in Fig. 1B and 1C shows a layer below the epithelium of densely packed 0.5-1.0-µm, rod shaped cells. Cell morphology is consistent with the identification of an *Enterovibrio* symbiont within *C. couesii* escae (Hendry *et al*, 2018; Baker *et al.,* in review). The bacterial cells are arranged in a layer between the epithelium and a layer of organic crystals that are characteristic of anglerfish escae (Pietsch 2009). (Fig.1C). The crystals were identified as organic as there no signal detected during elemental analysis using EDS. Crystals in the esca of anglerfish have been previously suggested important to the amplification of bioluminescence (Pietsch 2009) (Fig. 1D and 1E).

Analysis of Anglerfish Microbiome

Bacterial characterization of the anglerfish microbiome was done by comparing anglerfish organ types to the surrounding water. Significant differences were found in the microbial community richness and diversity between anglerfish and water samples (Fig. 2). The observed richness (ANOVA, df=7, F=65.91, p=<0.001) and Chao1 index (ANOVA, df=7, F=57.82, p=<0.001) showed significant differences in richness and diversity among sample types. Diversity as measured by the Shannon index (ANOVA, df=7, F=52.95, p=<0.001) and Inverse Simpson index (ANOVA, df=7, F=10.95, p=<0.001) also showed significant differences among sample types. NMDS visualization of the data revealed a distinct clustering of water samples while all anglerfish organ types overlapped (Fig. 3). Permuted multivariate ANOVA (Adonis) analysis showed that comparison of anglerfish specimens at the organ level to water provided a slightly greater explanation, accounting for 13% of the

variation between microenvironments (PERMANOVA, df=7, F=6.2828, R^2=0.126, p=0.001). A comparison of the anglerfish microbiome (including all tissue-types) and water using SIMPER revealed that water and anglerfish microbiomes are 98.5% dissimilar from one another, with 182 ASVs significantly contributing to this difference. The most significant ASVs that drive this difference between anglerfish and water are *Moritella* sp. (8.2%), *Enterovibrio luxaltus* (5.2%), *Pseudoalteromonas* sp. (5.0%), and *Vibrio* sp. (4.3%).

When comparing the microbial richness and diversity of anglerfish organ types to one another, we found significant differences in the microbial community richness and diversity. These were found by comparing bacterial communities on different organ types as measured by the Shannon index (ANOVA, df=6, F=2.436 p=0.03) and Inverse Simpson index (ANOVA, df=6, F=3.348, p=0.0046). These significant results were driven by differences between the guts and esca (Inv. Simpson, Tukey's HSD P=0.003) as well as guts and skin (Inv. Simpson, Tukey's HSD P=0.002). Members of the Vibrionaceae were most abundant overall when accounting for all areas of the anglerfish sampled (Fig. 4). Members of the family Moritellaceae are present in highest abundance on the fins, skin, and guts while Pseudoalteromonadaceae is most abundant within escal and illicial organs (Fig. 4).

Symbiont Taxa in Anglerfish Specimens

Sequencing revealed twelve distinct ASVs of the family Vibrionaceae that were greater than 0.1% relative abundance. ASVs with greater than 10% relative abundance in the light organs had top BLAST results (> 98%) were previously identified anglerfish symbionts; two ASVs matched "*Candidatus* Enterovibrio escacola" by greater than 98% and three ASVs matched "*Candidatus* Enterovibrio luxaltus" greater than 99%. *E. luxaltus* ASVs were identified with a relative abudance greater than 10% in nine out of 11 escal samples from *C. couesii* adults (Fig. 5A) and in four of nine caruncle specimens with a relative

abundance ranging from 45.6% - 98.8% (all *C. couseii* hosts). The *E. escacola* ASVs were found in three escal specimens and a single caruncle sample belonging to hosts within the families Melanocetidae, Oneirodidae, and Ceratiidae families. For samples with *E. luxaltus* ASVs, one ASV was present at much higher abundance than the other, suggesting that one may be the result of sequencing error. However, for *E. escacola* both ASVs were present in roughly equal abundance and these maybe have been amplified from the two rRNA operons present in the *E. escacola* genome (Hendry et al., 2018), in which the 16S rDNA sequences differ.

In addition to symbiont ASVs, eight ASVs were identified as five unique *Vibrio* ASVs and two *Photobacterium* ASVs. These ASVs were found on a variety of tissue types, but they were largely absent from non-light organ tissues (<10% relative abundance). Further investigation using BLAST was not able to distinguish these ASVs beyond genus level due to similarities in 16S rDNA for these genera.

Anglerfish symbiont ASVs matching the confirmed symbiont sequences were found in the majority of the anglerfish light organ samples, with the exception of two *Ceratias* esca, two *C. couesii* esca, and three *C. couesii* caruncle specimens. This may be due to anglerfish ejection of the symbiont after capture, a relative overabundance of skin-associated bacteria in these samples, or a relatively low contribution of anglerfish symbionts to 16S rDNA amplicons due to their low rRNA operon number (Hendry et al., 2018). Confirmed symbiont *E. luxaltus* ASVs were identified in other tissue types besides the light organs, although generally at lower relative abundance and in lower proportion of individuals than for light organ samples

Sequencing of larval anglerfishes revealed four additional Vibrionaceae ASVs, none of which matched confirmed symbiont sequence. These tissues did host two

Photobacterium sp. and five *Vibrio* sp. ASVs. None of the confirmed symbiont ASVs were found within the larval anglerfish specimens (Fig. 5A).

Symbiont Taxa in Seawater Samples

Confirmed *E. luxaltus* and *E. escacola* symbiont ASVs were detected at at low relative abundance in 13% of the seawater samples. The *E. luxaltus* ASVs, matching the symbiont associated with *C. couseii*, were found in 13% of samples at 0.003 - 0.415% abundance. The *E. escacola* ASVs were less prevalent; only one symbiont ASV was found in water samples and it was found in a single seawater sample at 0.035% abundance. Both anglerfish symbionts were found together in a single sample. When examined by depth, symbiont ASVs had on average the highest relative abundant within the mesopelagic zone, which was also true of the absolute abundance of confirmed symbiont ASVs (Fig 6).

DISCUSSION

Anglerfish and Seawater Microbiomes

Not unlike the findings of prior studies on fish-associated microbiomes and their environment (Larsen *et al.* 2015; Legrand *et al.* 2018; Pratte *et al.* 2018), there exists a significant difference in the richness and diversity of the host associated microbial community found within anglerfish body tissue specimens and the surrounding environment (Fig, 2). This pattern of microbiome distinctness is now a common phenomenon of symbiosis among many organisms in various ecosystems (McFall-Ngai et al, 2013; Thompson et al, 2017). In the anglerfish the greatest difference between body tissues and seawater communities is the greater abundance of the genera *Moritella*, *Pseudoalteromonas*, *Enterovibrio*, and *Vibrio* within anglerfish specimens. A single ASV from the genus *Moritella* was present at high relative abundance within most organs of adult anglerfishes. The Silva database identified this *Moritella* taxon as unidentified. Members of

the genus *Moritella* are generally piezophilic and are often associated with deep-sea organisms (Urakawa 2013). One member of the genus, *M. viscosa*, is known to cause skin ulcerations in fishes (Urakawa 2013), although several other species are not known to be pathogens and may be commensals or mutualists. Also present at high abundance levels on various types of tissues of adult anglerfishes were ASVs from the genus *Pseudoalteromonas*. There were twelve unique ASVs found associated with adult anglerfishes, eight of which were found on over half of the samples documented in this study. Known members of *Pseudoalteromonas* have been reported to provide antifouling and/or algicidal benefits (Holmström and Kjelleberg 1999). More detailed investigation may determine if the taxa identified here also exhibit antifouling properties, which may aid the host in reducing the presence of microbes that may compete with or prevent colonization by other anglerfish-associated microbes, such as mutualists or pathogens.

Microbiomes of adult anglerfishes

Examining adult anglerfish specimens by organ type did not reveal any significant differences in microbial richness or diversity. However, the escae and caruncles of adult anglerfishes had the lowest levels of microbial richness and diversity in comparison to other organ types sampled, which is predicted given the likely monoculture of symbionts within the light organs (Hendry et al., 2018). The lack of significant difference was likely due to the fact that the entire bioluminescent organ was processed, including the outer epithelial surface, which could inflate the apparent diversity and richness of these organs.

Bray-Curtis dissimilarity analysis revealed that the collection site (station) accounted for the greatest amount of variation seen within adult anglerfish specimens. This was primarily driven by the high abundance of *Moritella* sp. present in samples collected from stations SW5 and B175. However, samples were unevenly sampled across stations, so

it is difficult to draw any strong conclusions. Host species accounts for the second-highest portion of variation seen within adult anglerfish microbial communities. Several previous studies have indicated that host species plays a significant role in the microbial community of fishes (Larsen *et al.* 2013; Boutin *et al.* 2014; Pratte *et al.* 2018). These findings indicate that the microbiome of adult anglers is influenced in part by the environment, but may also be regulated by host-specific relationships with microbes which are currently not fully understood.

Adult anglerfish bioluminescent symbionts

Whole bioluminescent organs of adult anglerfishes were dominated by twelve Vibrionaceae ASVs. Vibrio species were present at fairly high abundance levels within nonluminous organ types and were not found abundantly in metagenomic sequencing of these nonluminous organs, so we conclude that most of these are likely associated with the skin of the light organs. In contrast, the five ASVs that were >10% relative abundance had greater than 98.5% similarity to symbiont genome assemblies containing luminescence genes from light organs (Hendry et al., 2018) and are assumed to be the luminous symbionts of these samples. Our results support a host-species specific symbiotic relationship between *C. couesii* hosts and symbiont ASVs, which are 99% similar to the 16S sequence of "*Candidatus* Enterovibrio luxaltus" (Hendry *et al.*, 2018; Baker *et al*., in review). Previous full genome sequencing of the *M. johnsonii* bioluminescent symbiont indicates a second symbiont species, "*Candidatus* Enterovibrio escacola" is 99% similar to other symbiont ASVs (Hendry *et al.* 2018). Here, two *E. escacola* symbiont ASVs were found in the escal bacterial communities of Melanocetidae, one *Oneirodes* sp. , and an unknown *Ceratias*. These findings corroborate genomic sequencing results demonstrating a specific relationship between *C. couesii* and "*Candidatus* Enterovibrio luxaltus" and the sharing of "*Candidatus* Enterovibrio escacola" among other anglerfish species (Baker et al., *in review*).

For the *C. couesii* specimens from which a caruncle and escal specimen were collected, the "*Candidatus* Enterovibrio luxaltus" ASVs appeared in high abundance within both organ types. This confirms prior observations of bioluminescent bacteria possibly oozing from the caruncles of freshly collected specimens (Pietsch 2009) and indicates that the same symbiont taxon is cultivated by the host in both esca. It has also been hypothesized that the illicium may provide a way for the bioluminescent symbiont to be transferred from the caruncle to the esca (Pietsch 2009), but symbiont ASVs were not identified at high abundance levels within the illicia of adult *C. couesii* individuals. Since "*Candidatus* Enterovibrio luxaltus" ASVs were not detected at >0.1% relative abundance within the illicia of any *C. couesii* specimen for which an escal and caruncle specimen were also processed, the illicium likely does not provide a continuous means for symbiont transport between the caruncle and esca of adult *C. couesii*.

Microbial communities and symbionts of larval anglerfishes

As in adults, collection location (station) explained the greatest portion of variation within the microbial communities of larval anglerfishes. However, collection depth was the second-strongest driver of beta diversity. Unfortunately, due to the nature of sample collection, a large portion of larval specimens were collected from net N0, which collected samples throughout the entire descent from the surface to the maximum depth of 1500m so we are unable to discern at which discrete depth the specimen was collected during DEEPEND cruises DP03 and DP04. These samples were binned together and thus reduced the strength of this observation.

Of the 13 larval samples included here, none were identified to host the confirmed symbiont ASVs. The Vibrionaceae found associated with larval samples were *Photobacterium* and *Vibrio* ASVs but were not the confirmed symbiont ASVs. It should be

noted that these larvae were collected at depths between 10 m and 999 m so it is possible that the larvae had already begun their ontogenetic vertical migration and had yet to acquire symbionts. If these larvae had been indirectly inoculated by the mother through her egg veil (Haygood et al 1993; Pietsch 2009), the relative abundance of symbiont ASVs in the larval specimens would have to be less than the relative abundance symbiont ASVs within seawater samples (0 – 0.66%). Therefore, the lack of symbiont ASVs in larval samples suggests that larvae do not have abundant symbionts, as would be expected with vertical transmission.

Bioluminescent symbionts within seawater

To examine the possibility that the larvae may be acquiring symbionts from their environment, we searched for the potential symbionts within seawater samples. Multiple sequences of the two anglerfish symbiont ASVs were found within the water at very low levels of relative abundance. This finding may imply that the bioluminescent symbionts of ceratioids are not obligately dependent for growth as they are able to survive outside of the host and therefore are more likely to be acquired from the environment. These findings are supported by the full genome analysis of the bioluminescent symbionts from *C. couesii* and *M. johnsonii* specimens, which indicated that these symbionts have retained cell-wall formation and motility genes (Hendry *et al.*, 2018), and a comparison of host and symbiont phylogenies which concluded that these partners are not codiverging, as occurs with vertical transmission (Baker *et al*., *in review*). In addition, both symbionts were found at the greatest abundance within the mesopelagic and bathypelagic zones. A greater concentration of these ASVs at these depths supports the idea that larval anglerfishes acquire bioluminescent symbionts from the environment as the esca develops during the larvae migration from the surface waters to the bathypelagic zone (Pietsch 2009). Moreover, many examples exist for environmental symbiont acquisition: several species of fish acquire their

luminous symbionts from environmental populations (Dunlap and Urbanczyk 2013) and the Hawaiian bobtail squid, *Euprymna scolopes,* carries out an elaborate selection process to procure their bioluminescent bacterium *Aliivibrio fischeri* from sea water (Nyholm and McFall-Ngai, 2004). Similarly, deep-sea *Osadex* worms acquire their important bone-digesting Oceanospirillales bacterial endosymbionts from the environment after every unique settlement onto decaying whale carcasses (Rouse et al, 2009).

CONCLUSIONS

This study is the most comprehensive analysis to date of the ceratioid microbiome and symbionts via molecular methods. Examining the microbial community present within the luminous esca, caruncle, other organs and larval anglerfishes in addition to seawater collected from the Gulf of Mexico revealed that ceratioid bioluminescent symbionts are present in seawater. However confirmed bioluminescent symbionts were not found from larval specimens, supporting the notion of absence throughout host development. Rather anglerfishes may acquire symbionts from the environment, as has been suggested by other studies (Baker *et al*, in review). Future sequencing studies would be beneficial in determining the importance of prevalent anglerfish-associated microbes such as *Moritella* and *Pseudoalteromonas* in influencing anglerfish health and symbioses.

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Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (R4.x257.230:0004, R4.x257.230:0001, R4.x257.228:0001. Anglerfish sequences from DP01-DP04 have also been deposited to the NCBI Short Read Archive (#PRJNA514914)

Supplemental Material

http://www.deependconsortium.org/images/documents/DP01_report.pdf,
http://www.deependconsortium.org/images/documents/DP02_CruiseReport.pdf,
http://www.deependconsortium.org/images/documents/DP03_CruiseReport.pdf, and
http://www.deependconsortium.org/images/documents/DP04_Cruise_Report.pdf.

Supplemental Tables

Supplemental Table 1 – Illumina Sequencing Statistics.

Supplemental Table 2 - Water samples collected for microbiome analysis (Triplicates were collected for each sample).

Supplemental Table 3 – ADONIS analysis

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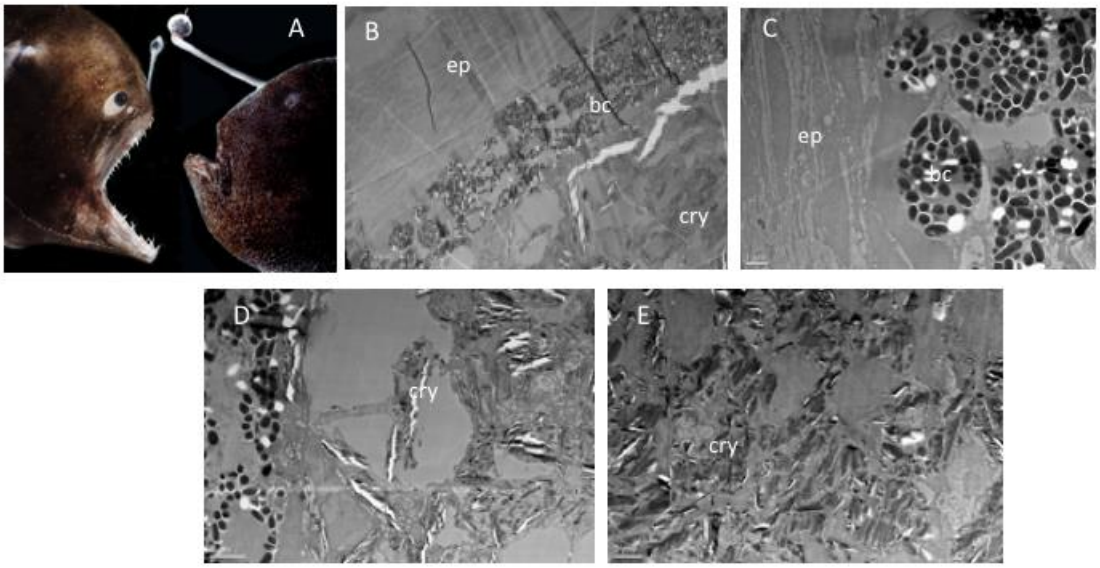

Figure 1A. A *Melanocetus johnsoni* individual is shown on left, while a *Cryptopsaras couesii* sample is on the right

Figure 1B Electron micrographs of layers within a *Cryptopsaras couesii* esca include an outer layer of epithelium tissue (ep), beneath this is a layer of densely packed cells containing bacteria (bc), and an inner layer of organic crystals (cry).

Figure 1C. Epithelium tissue (ep) with individual bacteriocytes containing symbiont cells (bc)

Figure 1D. The lower portion of the bacteriocytes (bc) showing contact with the layer of organic crystals (cry) in the center of the esca.

Figure 1E. Random orientation of the presumably organic crystals (cry) in the center of the esca.

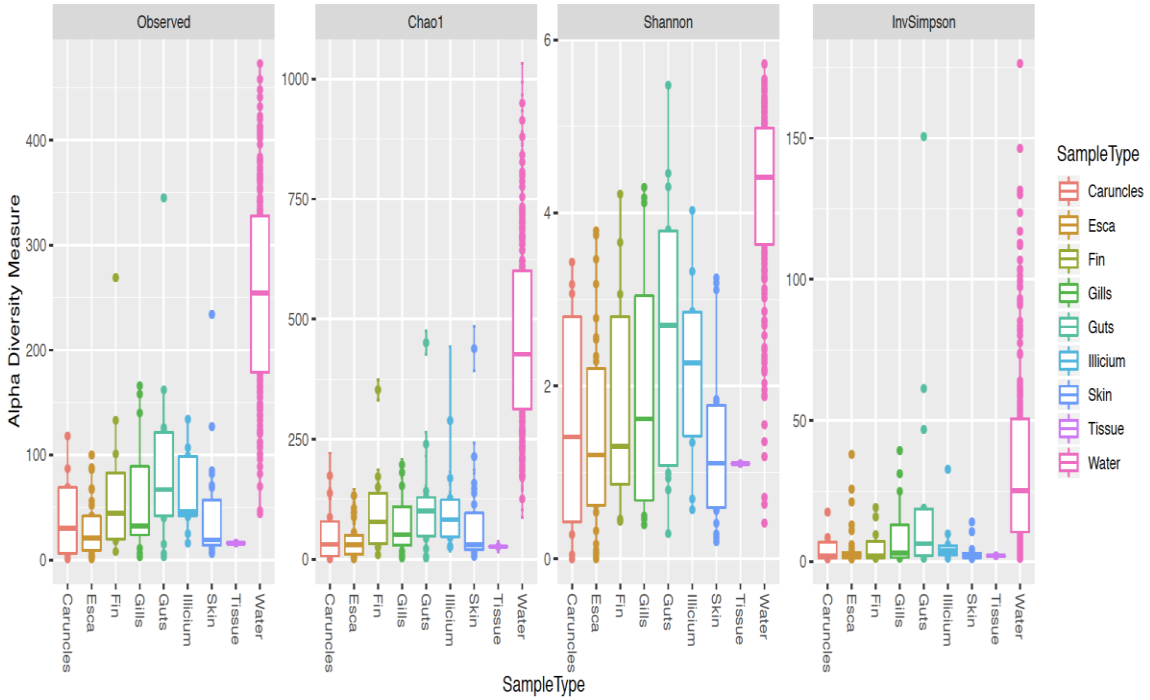

Figure 2. Boxplot of species richness and diversity comparing sample types based on observed richness (ANOVA, df=7, F=68.15, p=<0.001), Chao1 index (ANOVA, df=7, F=40.76, p=<0.001), Shannon index (ANOVA, df=7, F=89.5, p=<0.001), and Inverse Simpson index (ANOVA, df=7, F=20.51, p=<0.001).

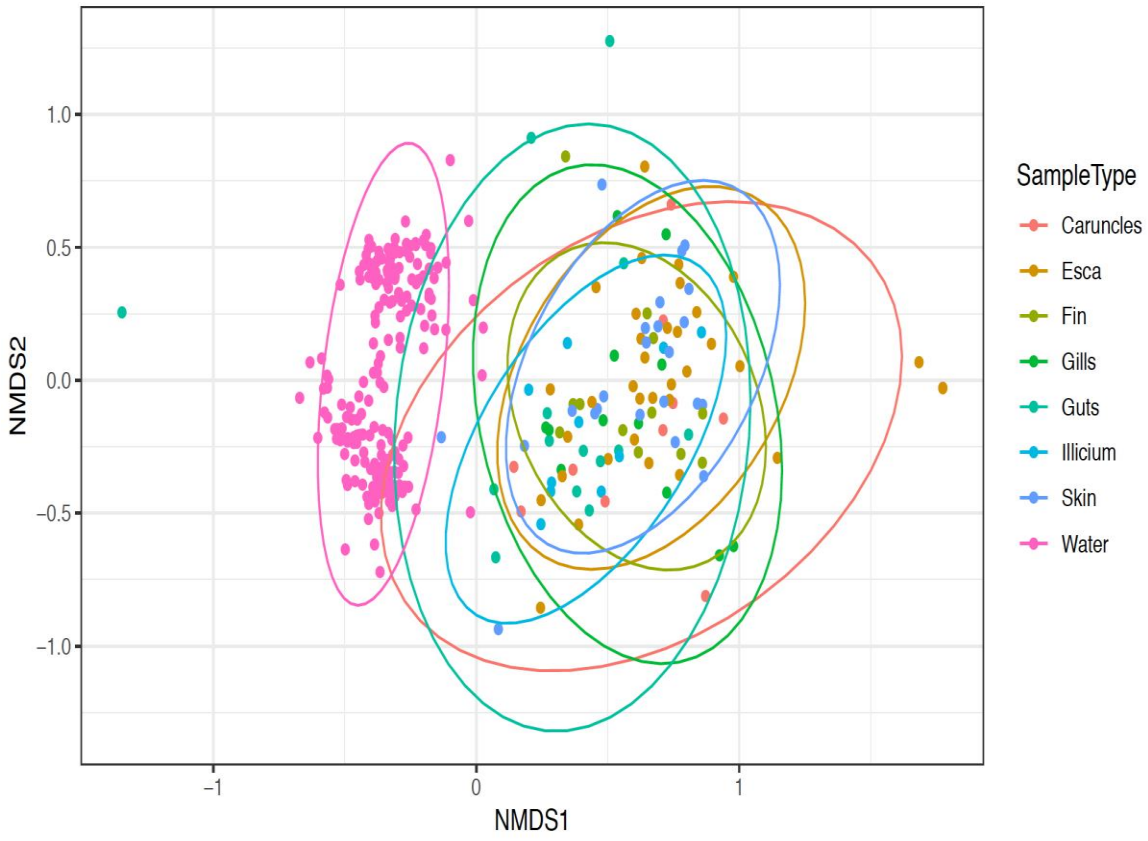

Figure 3. Non-metric dimensional scaling of anglerfish and water samples. (R^2 = 0.97, stress= 0.1699, solid ellipse = multivariate normal distribution with 95% CI).

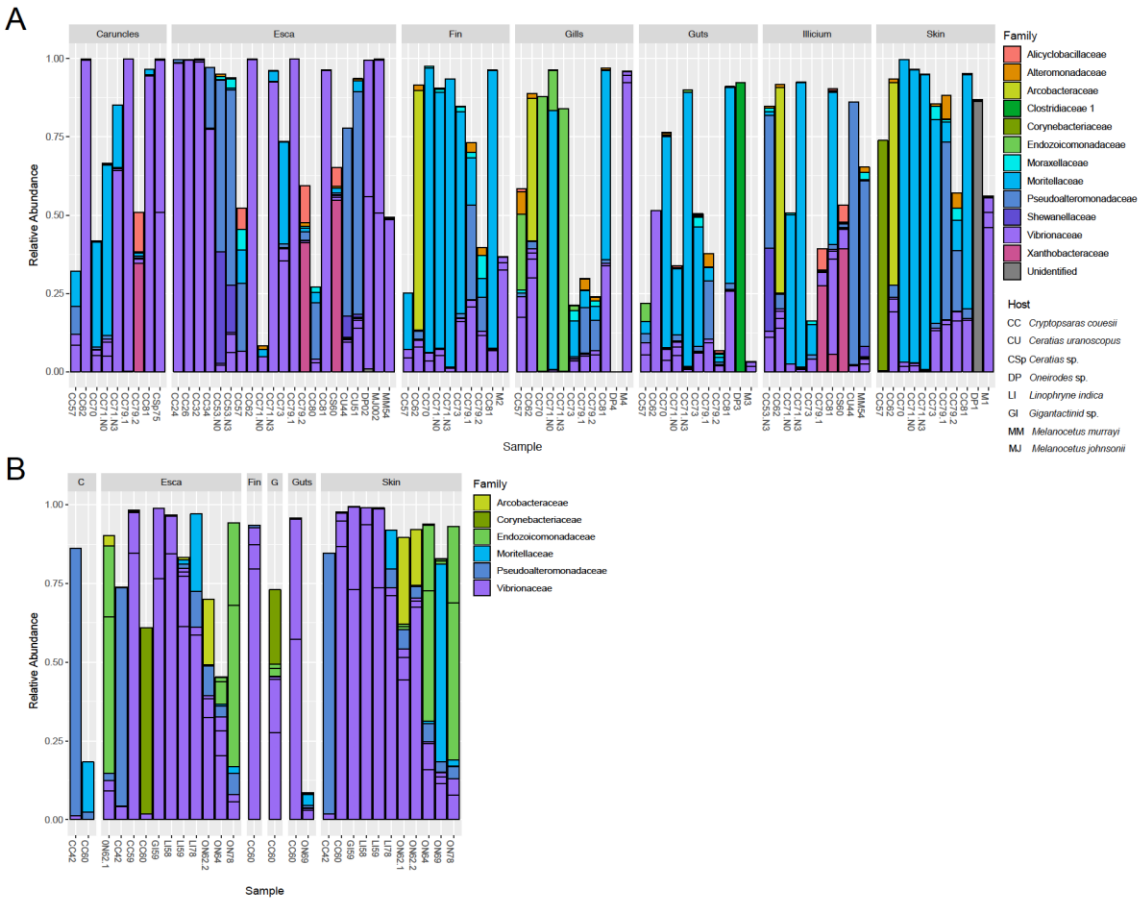

Figure 4A. Bar plot of taxa present at greater than 0.1% relative abundance within adult anglerfish specimens by Family.

Anglerfish taxa are abbreviated as follows - *Cryptopsaras couesii (CC), Melanocetus johnsonii (MJ), Melanocetus murrayi (MM), Centrophryne spinulosa* (CSp), unknown Linophrynidae sp. (L), undefined *Ceratias* sp. (CU), unknown *Oneirodes* sp. (O), unknown *Gigantactinidae* sp (G)

Figure 4B. Bar plot of taxa present at greater than 0.1% relative abundance within larval anglerfish specimens by Family.

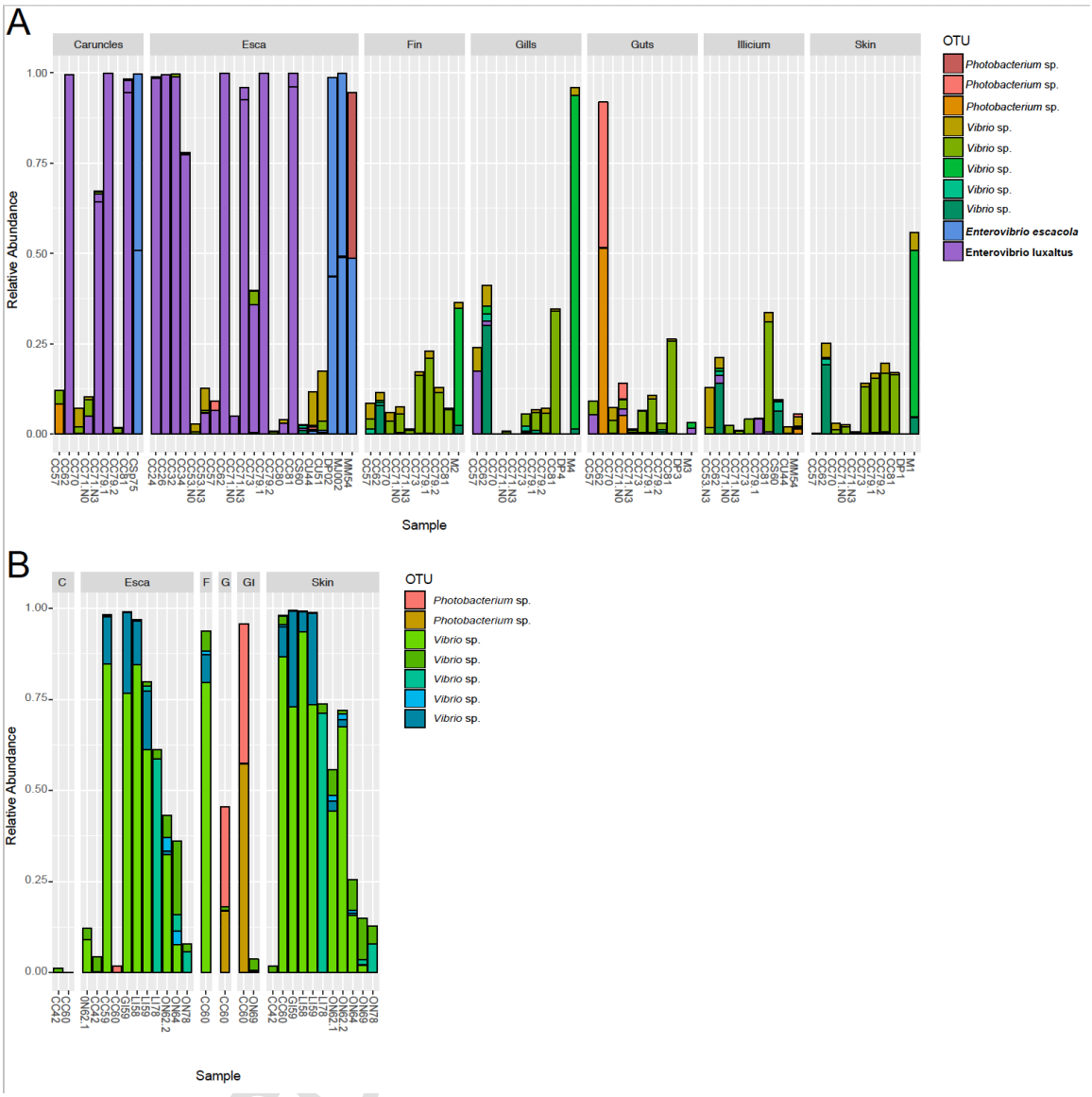

Figure 5A. Bar plot of Vibrionaceae ASVs within adult anglerfish specimens. **Anglerfish taxa are abbreviated as in Figure 4.**

Figure 5B. Bar plot of Vibrionaceae ASVs within juvenile anglerfish specimens.

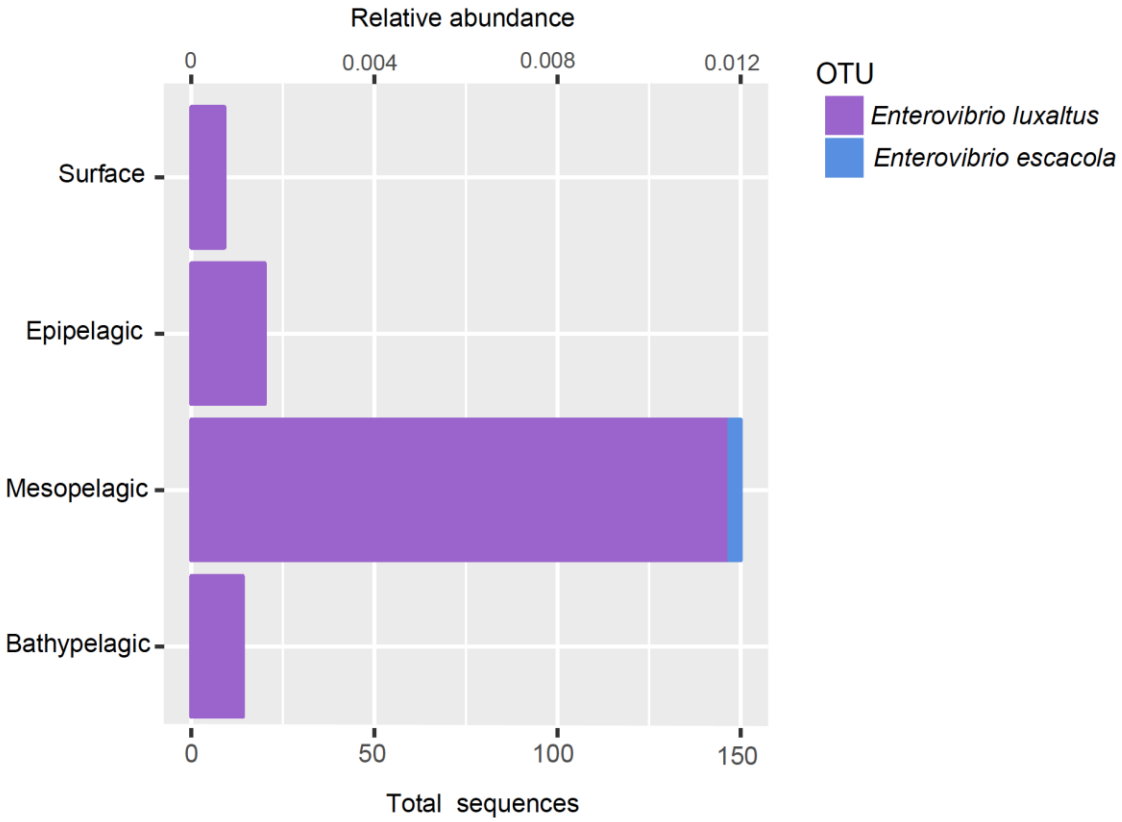

Figure 6. Relative abundance of potential symbiont ASVs in seawater by Depth Zone. A total of 220 seawater samples (combining replicates) were analyzed. These samples were also described by Easson and Lopez (2019). Twenty-seven total samples were positive for *E. luxaltus* ASV and only 1 was positive for *E. escacola*.

Table 1. Anglerfishes collected for microbiome analysis. Abbreviations for sampled organs: caruncle (c), esca (e), fins (f), illicium (i), gills (g), guts (gu), and/or skin (s).

ID	Taxonomy (Family, species)	Dev. Stage	Organs sampled	Cruise	Station	Trawl #	Trawl Depth (m)
DP02	Oneirodidae *Oneirodes* sp.	Adult	e, g, gu, s	DP01	B001	02	0-1201
MJ02	Melanocetidae *Melanocetus johnsonii*	Adult	e, f, g, gu, s	DP01	B001	03	0-1143
CC24	Ceratiidae *Cryptopsaras couesii*	Adult	e	DP02	B252	24	600-198
CC26	Ceratiidae *Cryptopsaras couesii*	Adult	e	DP02	B080	26	0-751
CC32	Ceratiidae *Cryptopsaras couesii*	Adult	e	DP02	SE3	32	597-198
CC34	Ceratiidae *Cryptopsaras couesii*	Adult	e	DP02	B255	34	1000-600
CC42	Ceratiidae *Cryptopsaras couesii*	Larva	c, e, s	DP03	B003	42	998-599
CC53.N0	Ceratiidae *Cryptopsaras couesii*	Adult	e	DP03	B081	53	11-1504
CC53.N3	Ceratiidae *Cryptopsaras couesii*	Adult	e, i	DP03	B081	53	1002-601
CU44	Undefined *Ceratias* sp.	Adult	e, i	DP03	B079	44	997-601
CU51	Undefined *Ceratias* sp.	Adult	e	DP03	B252	51	11-1502

MM54	Melanocetidae *Melanocetus murrayi*	Adult	e, i	DP03	B081	54	11-1500
CC57	Ceratiidae *Cryptopsaras couesii*	Adult	c, e, f, g, gi, s	DP04	SW6	57	10-924
LI58	Unknown Linophrynidae sp.	Larva	e, s	DP04	SW6	58	1515-1203
CC59	Ceratiidae *Cryptopsaras couesii*	Larva	e	DP04	SW6	59	202-10
GI59	Unknown Gigantactinidae sp.	Larva	e, s	DP04	SW6	59	10-1500
LI59	Unknown Linophrynidae sp.	Larva	e, s	DP04	SW6	59	1498-1201
CC60	Ceratiidae *Cryptopsaras couesii*	Larva	c, e, f, g, gu, s	DP04	SW4	60	999-602
CS60	Centrophrynidae *Centrophryne spinulosa*	Adult	e, i	DP04	SW4	60	999-602
ON62.1	Unknown Oneirodidae sp.	Larva	e, s	DP04	SE1	62	11-1499
CC62	Ceratiidae *Cryptopsaras couesii*	Adult	c, e, i, f, g, gu, s	DP04	SE1	62	11-1499
ON62.2	Unknown Oneirodidae sp.	Larva	e, s	DP04	SE1	62	11-1499
ON64	Unknown Oneirodidae sp.	Larva	e, s	DP04	SE3	64	11-1501
ON69	Unknown Oneirodidae sp.	Larva	e, gu, s	DP04	SW3	69	998-601
CC70	Ceratiidae *Cryptopsaras*	Adult	c, f, g, gu,	DP04	SW5	70	998-600

	couesii		s				
CC71.N0	Ceratiidae *Cryptopsaras couesii*	Adult	c, e, f, g, gu, i, s	DP04	SW5	71	11-1505
CC71.N3	Ceratiidae *Cryptopsaras couesii*	Adult	c, e, f, g, gu, i, s	DP04	SW5	71	1001-593
CC73	Ceratiidae *Cryptopsaras couesii*	Adult	e, f, g, gu, i, s	DP04	B064	73	11-1512
ON76	Unknown Oneirodidae sp.	Post Larva	e, f, g, gu, s	DP04	B065	76	1000-599
LI78	Unknown Linophrynidae sp.	Larva	e, s	DP04	B287	78	996-603
ON78	Unknown Oneirodidae sp.	Larva	e, s	DP04	B287	78	11-1501
CC79.1	Ceratiidae *Cryptopsaras couesii*	Adult	c, e, f, g, gu, i, s	DP04	B252	79	1001-605
CC79.2	Ceratiidae *Cryptopsaras couesii*	Adult	c, e, f, g, gu, s	DP04	B252	79	1001-605
CC80	Ceratiidae *Cryptopsaras couesii*	Adult	e	DP04	B252	80	10-1500
CC81	Ceratiidae *Cryptopsaras couesii*	Adult	c, e, f, g, gu, s	DP04	B175	81	1000-600

Additional environmental data on each MOCNESS cast can be obtained from the appropriate DEEPEND cruise report (http://www.deependconsortium.org/)