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Stability, Development, and Function of a Symbiotic Bacterial Community Associated with the Reproductive System of the Hawaiian Bobtail Squid, Euprymna scolopes

Allison H. Kerwin
University of Connecticut, allison.kerwin@uconn.edu

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Allison Helen Kerwin, Ph.D.
University of Connecticut, 2017

Many aquatic organisms deposit their eggs into an environment where successful embryogenesis depends on minimizing biofouling. The female Hawaiian bobtail squid, *Euprymna scolopes*, harbors a diverse bacterial community within the accessory nidamental gland (ANG), a symbiotic reproductive organ. This community is hypothesized to be environmentally transmitted, and to be deposited from the ANG into the egg jelly coat (JC). Illumina sequencing of the 16S rRNA V4 gene region demonstrated that the ANG bacterial community (n=29) and that of the JC (n=35) were composed primarily of members of the *Rhodobacteraceae* and *Verrucomicrobia*, which together comprised on average 86% of the sequences recovered per sample (68% and 18% respectively). JC bacterial communities clustered with the ANG community of the female that produced those eggs, suggesting that bacteria from the ANG are deposited directly into the JC. OTUs representing 94.5% of the average ANG abundance were found in the natural squid environment, consistent with the hypothesis of environmental transmission between generations. The ANG bacterial community gradually changed from a *Verrucomicrobia*-dominated to an *Alphaproteobacteria*-dominated community over the course of host sexual development. The surface of the immature ANG was covered in microvilli and contained numerous ciliated invaginations, demonstrating that the immature ANG is poised for colonization by environmental bacteria. Eggs treated with antibiotics over the course of embryogenesis developed a biofilm, primarily composed of the fungus *Fusarium keratoplasticum*, which led to the death of the embryos (3% viability; n=17 clutches). Fungal

challenge experiments on dissected eggs demonstrated that the JC containing the bacterial community is essential for egg defense from fungal bud cells (n=3 trials, 8-10 eggs/treatment). Extracts from ANG/JC bacteria were also able to inhibit *F. keratoplasticum in vitro*. Taken together, these data suggest that the ANG/JC bacteria protect developing embryos from biofouling. Ongoing work is focused on identifying specific strains and compounds responsible for antifungal activity. This association offers a unique experimental model for understanding mechanisms by which marine invertebrates protect their eggs in the environment. This research has set the background for utilizing the *E. scolopes* ANG system as a model for studying how consortial symbioses are established and maintained.

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Allison Helen Kerwin

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Presented by

Allison Helen Kerwin, B.A., M.S.

Major Advisor_____
Spencer V. Nyholm

Associate Advisor_____
Joerg Graf

Associate Advisor_____
Jonathan Klassen

Associate Advisor_____
Daniel Gage

Associate Advisor_____
Nichole Broderick

University of Connecticut
2017

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Chapter 1

Introduction

Invertebrate and vertebrate animals rely on various modes of reproduction, including viviparity (live birth), and oviparity (egg-laying), to produce their young (Blackburn 1999). Oviparous strategies expose eggs to a wide variety of inherent threats. In aquatic/marine environments, eggs of both vertebrate and invertebrate marine animals may be vulnerable to biofouling, infection, and predation (Blackburn 1999). Embryogenesis, the time over which the embryo develops in the egg before hatching, can last for extended periods (weeks to months) in the marine environment compounding these vulnerabilities: for example, nudibranch egg embryogenesis lasts for 12 days (Carroll and Kempf 1990), while that of the American lobster, *Homarus americanus*, lasts for 9 months (Helluy and Beltz 1991). These vulnerabilities are compensated for by a wide variety of mechanisms. In some organisms, parental care fills this role with some parents putting all of their energy into protecting their eggs. In certain octopods, females guard their eggs 24 hours a day, flushing water over the clutch of eggs to prevent fouling and increase oxygenation, and refrain from feeding themselves with the result that the female may not survive embryogenesis (Boletzky 1986). In other organisms, the strategy instead is to produce numerous eggs, so that even if most are lost to predation, fouling, or infection, the small fraction that survive will be enough to continue the next generation (Parry 1981). Another strategy involves chemical defense of the eggs, either from host factors or from symbiotic partners (Flórez et al. 2015). Chemical protection can be embedded into the eggs prior to oviposition (egg-laying) or can be provided during embryogenesis by the developing embryo or by symbionts in the egg. Symbionts in the eggs can either come from the environment or can be deposited in the eggs by the mother.

Cephalopods have long been the focus of scientific research with many diverse goals. Their large neurons have made them a good model system for neuroscience, while their

camouflage abilities have made them a target for military research. Their reproductive behavior has also been the subject of research dating as far back as Aristotle, who observed:

> "The molluscs also breed in the spring. Of the marine molluscs one of the first to breed is the sepia. It spawns at all times of the day and its period of gestation is fifteen days." [Aristotle, 350 B.C.]

Cephalopods, like many other marine organisms, lay their eggs in seawater that contains millions of prokaryotic organisms in every milliliter, as well as thousands of algal and fungal spores, and various aquatic larvae (Goeke et al. 2010). In this type of an environment, biofilms form on unprotected surfaces very quickly, beginning within minutes to hours. However, many cephalopod eggs, specifically those of squid, bobtail squid, and cuttlefish, are not subjected to biofouling or predation throughout their development, which can last in some cases for weeks or even months (Fields 1965, Sauer and Smale 1993, Smale et al. 1995). Most of these species provide no parental care, so these eggs must rely on some other mechanism for protection (Fields 1965).

One such potential mechanism is found in a reproductive organ present in female squid, bobtail squid, and cuttlefish (Buchner 1965). This organ is called the accessory nidamental gland (ANG), and consists of numerous epithelium-lined tubules that contain a bacterial consortium.

Historical research on the ANG in cephalopods

Over the past century, the ANG came under investigation, although the function of this organ was a mystery for most of that time. In 1911, while examining the sexual activities of the market squid, *Doryteuthis pealeii*, Drew observed that when a female is ready for oviposition,

the borders of the ANG became very red and could be seen through the semi-transparent mantle (Drew 1911). In this same study, Drew discussed that both the ANG and nidamental glands secrete viscous materials which must be mixed as they encase the eggs (Drew 1911). Shortly after this study, it was reported that the ANG contained tubules which are full of bacteria, and that each tubule represents a pure culture of bacteria, or at least is dominated by one type (Pierantoni 1918). Pierantoni classified the ANG as a symbiotic organ, containing both bacilli (in white/yellow tubules) and cocci (red tubules) in *Sepia officinalis*, and bacilli (red tubules) and cocci (white tubules) in *Sepiola intermedia* (Pierantoni 1918). Another paper from this era discussed the development of the ANG in *Sepia elegans*, finding that epithelial thickening develops into a ridge system which then grows into invaginations and finally glandular tubules (Döring 1908). The rudimentary organ is shown to be covered with cilia, as are the invaginations, but the eventual tubules, which are filled with symbionts, lack as many cilia (Döring 1908). Döring also reported that the rudiments of the *S. elegans* and *S. officinalis* ANG turned a brownish-black color when the animal reached ~16mm mantle length, as the individual tubules were filled with sand and detritus, but that these foreign materials progressed towards the periphery and ducts. The cilia development coincided with cleansing and afterwards the tubules developed fully and formed a tangled network (Döring 1908). Döring also suggested that these materials could contain the organisms which eventually colonized the tubules. This observation of sand entering the tubules is the only report of such an event that exists in the literature, and as Döring was working with museum specimens, the possibility that this sand was a contaminant that occurred during the fixation process cannot be ruled out.

After the above studies, little work was done on the reproductive system of cephalopods for another 45 years. Subsequently, a review of cephalopod symbiotic organs was published,

revealing the state of knowledge at that time (Buchner 1965). Buchner evaluated the hypothesis that the ANG assists in construction of the eggshell (egg case or capsule), but determined that it does not, and as a result considered this organ to be an "accessory" gland. The Döring and Pierantoni papers were covered in detail in this review, as were conflicting reports in which the contents of the ANG tubules were hypothesized to be nuclei instead of bacterial symbionts. Buchner was the first to describe the full host range of the ANG: the loliginids and sepioids (Buchner 1965). Up to this point the ANG had been studied primarily in the sepioids, as well as in a few loliginids, but how widespread this symbiosis was in the loliginids remained undescribed (Buchner 1965). One notable point mentioned in this review, which to our knowledge remains unconfirmed, is the presence of a rudimentary ANG in *Loligo forbesi* males, located in front of the kidney openings, which eventually regresses, and in *Austrorossia mastigophora* males (Buchner 1965). *L. forbesi* is also unusual in that embryos are reported to have a rudimentary ANG prior to hatching (Buchner 1965).

Overall, this research from the early twentieth century provided a framework for more recent studies, which over the past forty years have begun to tease apart the host-microbe interactions of this symbiosis.

Structure of the ANG

The ANG is a secretory organ. Tubules have a single layer of epithelial cells containing rough endoplasmic reticulum and are covered on the lumen side in a continuous layer of dense microvilli, frequent patches of cilia, and specialized secretion structures (Bloodgood 1977). Morphological characterization of the epithelial cells lining the tubules demonstrated that two types of cells were present, depending on the bacterial population contained within the tubules: one epithelial type was vacuole-rich and appeared secretory, while the other type was more

electron dense, lacked vacuoles, and did not appear secretory (Collins et al. 2012). In *D. pealeii*, the ANG has a ventral duct which opens into the mantle cavity near the nidamental gland opening (Bloodgood 1977). Tubules with similar coloration have been found to cluster together in *D. pealeii* (Bloodgood 1977). Both morphological examination via transmission electron microscopy, and fluorescence *in situ* hybridization (FISH), have demonstrated that each tubule contains a single dominant bacterial taxon (Bloodgood 1977, Collins et al. 2012), a conclusion also reached much earlier by Pierantoni (1918).

Bacterial symbionts of the ANG

The bacterial composition of the ANG appears to depend largely on the host species examined. Many earlier studies depended on culture-based methods to elucidate the contents of the ANG, resulting in a bias towards *Gammaproteobacteria*, which tend to be easier to culture and which grow quickly (Lum-Kong and Hastings 1992, Barbieri 1996, 1997). These methods were then phased out, and replaced by FISH and by the preparation of 16S rRNA gene clone libraries (Barbieri et al. 2001, Pichon et al. 2005, Grigioni et al. 2000).

One of the most conclusive studies utilized both FISH and clone libraries to conclude that the *D. pealeii* ANG contained 65% *Alphaproteobacteria* and 5% *Gammaproteobacteria*, with the remainder of the community uncharacterized (Barbieri et al. 2001). An earlier study from the same group using culture-based methods identified *Alteromonas*, *Aeromonas*, *Vibrio*, and *Roseobacter* strains from the *D. pealeii* ANG (Barbieri et al. 1996). A culture-based study of the ANG symbionts of *L. forbesi* obtained only *Gammaproteobacteria*, namely *Vibrio* and *Pseudomonas* strains (Lum-Kong and Hastings 1992). A study which examined a wide variety of loliginid hosts found that the loliginid ANG contained bacteria from the *Agrobacterium-Silicibacter*, *Roseobacter*, *Rhodobacter*, *Gammaproteobacteria*, *Cytophaga-Flavobacteriia-*

Bacteroidetes, and *Clostridium* groups (Pichon et al. 2005), however, this study, while broad in scope, only examined a few clones per specimen, and thus failed to provide an overall picture of the community composition for any given species.

Outside of the loliginids only a few studies have examined ANG bacterial composition. The ANG from the pygmy squid, *Idiosepius*, was shown to contain members of the *Agrobacterium-Silicibacter*, *Rhodobacter*, and *Clostridium* groups (Pichon et al. 2005). The *S. officinalis* ANG bacterial community by contrast, was found to consist of a majority of Gram positive bacteria via light microscopy, although a concurrent clone library obtained mostly isolates related to *Agrobacterium* as well as a few *Rhodobium-Xanthobacter* strains (Grigioni et al. 2000).

Development and chemical nature of the ANG

The *S. officinalis* ANG changes over the course of maturation from colorless in immature animals, gradually to white, beige, yellow, orange and finally to a bright coral color in sexually mature animals (van den Branden et al. 1978). The red-orange pigment associated with the mature *S. officinalis* ANG was shown to be concentrated in "granules" at the center of ANG tubules (Decleir and Richard 1972). The illustrations provided of these granules appears to show the typical bacterial community within the ANG lumen. The pigment was analyzed and found to be a novel pigment, sepiaxanthine, which is hypothesized to form via the oxidation of yellow carotene also found in the ANG (Decleir and Richard 1972). ANG pigmentation has also been conclusively associated with the bacterial symbionts of *S. officinalis* and *D. pealeii* (van den Branden et al. 1978, Bloodgood 1977). Bacteria from the *S. officinalis* ANG were isolated, and the isolates' pigmentation examined, but sepiaxanthine was not found, suggesting that the

secretory activity of the ANG induces transformation of the pigment from adonixanthin (found in the bacterial isolates) to sepiaxanthine in the mature ANG (van den Branden et al. 1980).

This change in coloration is not restricted to *S. officinalis*. In *D. pealeii* the coloration of the ANG changes from white to bright red as squid approach spawning, hypothesized to be due to changes in bacterial metabolism and the luminal environment which affect pigment production (Bloodgood 1977). A similar change in coloration is also seen in *Sepiola atlantica*, *Loligo vulgaris*, and *Alloteuthis subulata* (van den Branden et al. 1978, Richard et al. 1979). These widespread and very obvious developmental changes have led to the use of ANG coloration in defining the stage of development of certain squid. For example, in *L. vulgaris reynaudii* the presence of an ANG is part of the definition of a squid reaching Stage II of maturity, and the increased coloration as the squid continue to mature continues to inform the various maturity stages (Lipinski and Underhill 1995).

The development of the cephalopod female reproductive system is a gradual process that does not begin for some time post-hatching. In *D. opalescens*, a rudimentary ANG is not present until at least 87 days post-hatching (Kaufman et al. 1998), while in *Sepia* sp. the ANG is not found until immature animals reach a mantle length of 9 mm (Döring 1908). ANG development has been most extensively studied in laboratory-raised *D. opalescens*, where a single epithelial cell layer formed adjacent to the ink sac, and was covered in microvilli and cilia (Kaufman et al. 1998). This epithelial layer formed ciliated invaginations open to the mantle cavity which were considered primordial tubules (Kaufman et al. 1998). This study unfortunately ended prior to the formation of full tubules but does appear to demonstrate that the *Doryteuthis* ANG is competent for bacterial colonization once this tissue begins to form. Immature *L. forbesi* ANGs (size/age not specified) also have invaginations at their surface, but were apparently further along in

development and also contained tubules deeper in the tissue, the size of which was inversely correlated with the distance from the surface epithelium (Lum-Kong 1992). As the female *L. forbesi* matured, the number and size of tubules increased and the epithelia of the tubules became secretory (Lum-Kong 1992). This research also found that the invaginations never completely vanished and that tubule formation continues throughout adulthood (Lum-Kong 1992), and is the only cephalopod we know of for which this claim has been made. Finally, the *S. officinalis* ANG has also been reported to have invaginations that then form into tubules (van den Branden et al. 1978).

ANG and egg jelly coat connection and structure

The placement of the ANG in close proximity to the nidamental glands has long linked these two organs together as playing some role in the formation of the egg capsule. The nidamental glands were first shown to produce the jelly coat material which encases the yolk sac and embryos of *D. pealeii* (Atkinson 1973). The jelly coat material is a complex mucopolysaccharide (Singley 1983), which contains a ciliary immobilizing factor that causes larval cilia to exhibit an asynchronous beat, potentially protecting eggs from ciliated predators (Atkinson 1973). This factor was isolated from crude viscous nidamental gland extracts, so the ANG bacteria were likely not responsible for this activity (Atkinson 1973).

While no bacteria were found in the embryo or yolk sac of either *L. vulgaris* or *Sepiateuthis lessoniana*, bacterial strains isolated from the egg capsule were the same as those found in the associated host ANGs (Pichon et al. 2005). Likewise, similar strains were found in both the ANG and egg capsule of *D. pealeii* and *E. scolopes* via FISH (Barbieri et al. 2001, Collins et al. 2012). The egg capsules of *D. opalescens* contain regular striations which were demonstrated to be high in protein (Biggs and Epel 1991). *D. opalescens* capsules are estimated

to contain 10^6-10^8 bacteria per capsule, with bacteria accounting for up to 10% of the capsule volume (Biggs and Epel 1991). Layers of capsule with dense bacterial growth alternate with layers that almost completely lack bacteria (Biggs and Epel 1991).

ANG-lacking cephalopods

While ANGs are found in many types of cephalopods, the *Octipodiformes*, *Nautiloida*, and, within the *Decapodiformes*, the *Oegopsida*, all lack ANGs (Lindgren et al. 2012). Octopods lack both an ANG and nidamental glands, and instead depend on the oviducal gland to produce any materials encapsulating the egg chorion (Boletzky 1986). Incirrate octopods have entirely replaced the protective function of encapsulation with active parental care by the female (Boletzky 1986).

Within the *Decapodiformes*, only the oegopsid squid lack ANGs, which is hypothesized to have been lost during the transition from a benthic to a pelagic lifestyle (Lindgren et al. 2012). Similar to the parental care found in incirrate octopods, the midwater squid, *Gonatus onyx*, protects its eggs by brooding throughout embryogenesis (Seibel et al. 2000).

The *Ommastrephidae* are a family of pelagic squid within the *Oegopsida*, and produce egg masses which are extremely watery jelly masses and lack any internal structure (Boletzky 1986), but these masses appear to have some inhibitory qualities. For example, the Humboldt squid, *Dosidicus gigas*, produces an aggregation of eggs in a watery gelatinous matrix that is resistant to microbial infection (Staaf et al. 2008). The egg mass of *Todarodes pacificus*, another ommastrephid squid, also has been shown to prevent crustaceans, protozoans, and bacteria from colonizing/infecting the eggs, although when the mass was damaged with the outer jelly disturbed or missing, the eggs were quickly infested by bacteria and protozoans (Bower and Sakurai 1996). However, the egg mass of a third ommastrephid, the short-finned squid *Illex*

illecebrosus, was found to suffer from extensive protozoan contamination which prevented the hatching of all but a fraction of the embryos (Durward et al. 1980), although as this experiment occurred in a laboratory setting, the egg mass may have been damaged.

While the *Ommastrephidae*, as part of the *Oegopsida*, are generally believed not to contain an ANG, two rather obscure studies have catalogued the ANG as present in the Humboldt squid, *D. gigas* (Báez 1980, Esperanza et al. 1986). In an effort to define maturity stages in *D. gigas*, ANG morphology was used to partially define the stage of maturity, and photographic evidence was provided (Báez 1980, Esperanza et al. 1986). Unfortunately, the black and white photographs included in these studies are not conclusive, and further evidence is needed to either confirm or refute the presence of an ANG within this species.

Function of the Symbiosis

The questions of why cephalopods maintain an ANG, and why females deposit ANG bacteria into their eggs, have been asked since the ANG was first described (Drew 1911, Pierantoni 1918, Williams 1909, Döring 1908). Many hypotheses have been proposed, including that the ANG contributes to formation of the "eggshell" (egg capsule, Buchner 1965, Drew 1911), and that the ANG material influences sexual maturation of the squid (Lum-Kong and Hastings 1992). Jelly coat bacteria were first hypothesized to serve a protective function in squid eggs in *D. opalescens* (Biggs and Epel 1991). This hypothesis has since been proposed for various species of cephalopods including *D. pealeii* (Barbieri 1997), *S. officianalis* (Grigioni 2000), *Loligo duvauceli* (Gomathi 2010), *L. vulgaris*, *S. lessoniana* (Pichon 2005), and *E. scolopes* (Collins et al. 2012). The hypothesis of this defensive function arose in part from the observation that *D. opalescens* eggs were not subject to animal predation or fungal or microbial

predation during embryogenesis, despite a complete lack of parental protection (Biggs and Epel 1991).

Despite the long-standing question of function, very little research has actually tested any of the above stated hypotheses. One study found that organic extracts from mature *L. duvauceli* ANGs had antibacterial activity against certain bacteria, including *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, but that extracts from immature ANGs and spent ANGs had no such activity (Gomathi et al. 2010). This difference in activity was attributed to the higher free fatty acid content of mature glands compared to immature glands, and more specifically to the higher proportion of unsaturated fatty acids, which had higher antibacterial activity than saturated fatty acids (Gomathi et al. 2010). Similar research found that organic, but not aqueous, extracts from three species of cuttlefish, *Sepia pharaonis*, *Sepia aculeata*, and *Sepiella inermis*, as well as the squid *L. duvaucelli*, inhibited both Gram negative (*E. coli*, *Aeromonas* sp., and *S. aureus*) and Gram positive (*Bacillus megaterium*) growth, although the level of inhibition varied by host species (Sherief et al. 2004). A study examining the antibacterial activity of *D. pealeii* found that while organic extracts of whole ANGs inhibited *Streptomyces griseus*, *Vibrio anguillarum*, and *Aeromonas salmonicida*, extracts of egg cases had no antibacterial activity (Barbieri et al. 1997). While intriguing, none of these studies directly tied this function to the bacterial symbionts, and did not demonstrate that this bacterial inhibition would be beneficial to the host. Barbieri and colleagues (1997) did test extracts from certain *D. pealeii* ANG bacterial isolates, and found antibacterial activity in *Shewanella* and *Alteromonas* isolates, but not in *Roseobacter* isolates.

Recently a more in-depth study characterized the antibacterial inhibition of an *E. scolopes* jelly coat isolate, *Leisingera* sp. JC1 (Gromek et al. 2016). This jelly coat symbiont

differentially inhibited certain marine vibrios, and the level of inhibition was shown to be affected by the concentration at which the potential pathogen was presented, with a higher level of inhibition shown for lower vibrio concentrations, possibly relating to the environmental concentrations at which potential bacterial pathogens are likely to be encountered (Gromek et al. 2016). *Leisingera* sp. JC1 was able to produce indigoidine, siderophores, and homoserine lactones, all of which could potentially play an integrated role in bacterial inhibition (Gromek et al. 2016).

This evidence together demonstrates that certain ANG bacterial symbionts are able to inhibit certain bacterial strains *in vitro*, but no evidence has been published to date demonstrating the function of this community *in vivo*, or to demonstrate that any of these potential bacterial pathogens are capable of infecting or harming the host.

Euprymna scolopes

The Hawaiian bobtail squid, *E. scolopes*, is endemic to the Hawaiian archipelago (Singley 1983), where it is consistently collected from two main sites on Oahu. Eggs have rarely been reported but appear to be deposited on coral ledges adjacent to sandy areas (Singley 1983). These bobtail squid rely on a binary symbiosis with the bioluminescent bacterium *Vibrio fischeri*, which helps the squid to avoid predation. This light organ symbiosis has served as a model for studying numerous beneficial host-microbe interactions, including quorum sensing, host immune response to beneficial and environmental microbes, and symbiont specificity (McFall-Ngai, 2014; Nyholm & Graf, 2012; Miyashiro & Ruby 2012; Nyholm & McFall-Ngai, 2004).

Recent investigations have also focused on the ANG bacterial community of *E. scolopes,* which is unique among the cephalopods in containing a large contingent of *Verrucomicrobia*

(Collins et al. 2012). The ANG community was shown to be dominated by *Alphaproteobacteria* (from the *Rhodobacterales* and *Rhizobiales* orders, 73%), with *Verruocomicrobia* and *Flavobacteriia* making up the majority of the remainder (17% and 11% respectively), using next generation sequencing techniques (Collins et al. 2012). Fluorescence *in situ* hybridization (FISH) demonstrated that some members of the ANG bacterial community are also present in the *E. scolopes* egg jelly coat (JC, Collins et al. 2012). However, the exact composition of the bacterial community in *E. scolopes* eggs and a comparison between the egg and ANG bacterial communities from corresponding individual females has not been reported.

A recent study examining the genomic potential of twelve *Alphaproteobacteria* isolates from the *E. scolopes* ANG representing the *Leisingera*, *Ruegeria*, and *Tateyamaria* genera, found that these strains have the potential for secondary metabolite production, including acyl homoserine lactones and siderophores (Collins et al. 2015). All isolates tested were able to produce detectable homoserine lactones and siderophores (Collins et al. 2015). All but one strain possessed a Type VI secretion system (T6SS, Collins et al. 2015). The T6SS was especially of interest as this structure could be involved in host-microbe interactions (Jani and Cotter 2010).

Research objectives

The cephalopod reproductive symbiosis found in the ANG has been the subject of scientific research for well over a century. Most of this research has depended on microscopy and culture-based techniques to draw conclusions about the organ, and as such has been fairly limited to more observational studies. Only recently have advancements in sequencing technology, and the ability to maintain *E. scolopes* in the lab, come together and allowed us to thoroughly examine all aspects of this symbiosis. My research attempts to combine in-depth and specific examination of individual ANG and jelly coat bacterial community composition with

ecological studies and natural product discovery to thoroughly characterize various aspects of the ANG symbiosis. Here I present my findings on the conservation of the ANG symbiosis within and among populations of *E. scolopes* and among different species of cephalopods, on the function of the symbiotic community, and on the development of the symbiosis from both host and symbiont perspectives.

Chapter 2

Symbiotic bacteria associated with a bobtail squid reproductive system are detectable in the environment, and stable in the host and developing eggs[+]

[+]Kerwin AH and Nyholm SV (2017) Symbiotic bacteria associated with a bobtail squid reproductive system are detectable in the environment, and stable in the host and developing eggs. Env Microbiol 19:1463-1475.

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Symbiotic bacteria associated with a bobtail squid reproductive system are detectable in the environment, and stable in the host and developing eggs

Allison H. Kerwin and Spencer V. Nyholm*
Department of Molecular and Cell Biology, University of Connecticut, CT 06269, USA.

Summary

Female Hawaiian bobtail squid, *Euprymna scolopes*, have an accessory nidamental gland (ANG) housing a bacterial consortium that is hypothesized to be environmentally transmitted and to function in the protection of eggs from fouling and infection. The composition, stability, and variability of the ANG and egg jelly coat (JC) communities were characterized and compared to the bacterial community composition of the surrounding environment using Illumina sequencing and transmission electron microscopy. The ANG bacterial community was conserved throughout hosts collected from the wild and was not affected by maintaining animals in the laboratory. The core symbiotic community was composed of *Alphaproteobacteria* and *Opitutae* (a class of *Verrucomicrobia*). Operational taxonomic units representing 94.5% of the average ANG abundance were found in either the seawater or sediment, which is consistent with the hypothesis of environmental transmission between generations. The bacterial composition of the JC was stable during development and mirrored that of the ANG. Bacterial communities from individual egg clutches also grouped with the ANG of the female that produced them. Collectively, these data suggest a conserved role of the ANG/JC community in host reproduction. Future directions will focus on determining the function of this symbiotic community, and how it may change during ANG development.

Received 19 August, 2016; revised 7 December, 2016; accepted 2 January, 2017. *For correspondence. Email: spencer.nyholm@uconn.edu; Tel. (860) 486-4886; Fax: (860) 486-4331. Sources of Support: This research was funded by NSF IOS-1557914 and the University of Connecticut to SVN. Conflict of interest: The authors declare no conflict of interest.

Introduction

A number of host-microbe interactions rely on symbiotic bacteria that are environmentally transmitted each generation. In marine ecosystems, cephalopods (loliginids, sepiids and sepiolids, Buchner, 1965) form symbioses with bacterial consortia that are associated with a specialized organ of the female reproductive system called the ANG. The ANG is made up of epithelium-lined tubules that house bacterial symbionts (Fig. 1, Bloodgood, 1977; Collins *et al.*, 2012). Although this bacterial association has been recognized for a century (Pierantoni, 1918), the function of the ANG remains largely uncharacterized. Published studies from two cephalopod species suggest that bacteria are deposited into the egg cases (Kaufman *et al.*, 1998; Collins *et al.*, 2012), where they are hypothesized to play a role in egg defense (Biggs and Epel, 1991). The dominant bacterial taxa of cephalopod ANGs are generally a combination of *Alphaproteobacteria, Gammaproteobacteria* and *Verrucomicrobia*, depending on the host species analysed (Barbieri *et al.*, 2001; Grigioni *et al.*, 2000; Pichon *et al.*, 2005; Collins *et al.*, 2012).

The bobtail squid, *Euprymna scolopes*, is endemic to the Hawaiian archipelago and lives in symbiosis with the bioluminescent bacterium *Vibrio fischeri*. This light organ symbiosis has served as a model for studying numerous beneficial host-microbe interactions, including quorum sensing, host immune response to beneficial and environmental microbes, and symbiont specificity (Nyholm and McFall-Ngai, 2004; Nyholm and Graf, 2012; Miyashiro and Ruby 2012; McFall-Ngai, 2014). Recent investigations have also focused on the ANG bacterial community of *E. scolopes*, which is unique among the cephalopods in containing a large contingent of *Verrucomicrobia* (Collins *et al.*, 2012). Fluorescence *in situ* hybridization (FISH) demonstrated that some members of the ANG bacterial community are also present in the *E. scolopes* egg JC (Collins *et al.*, 2012). However, the exact composition of the bacterial community in *E. scolopes* eggs and a comparison between the egg and ANG bacterial communities

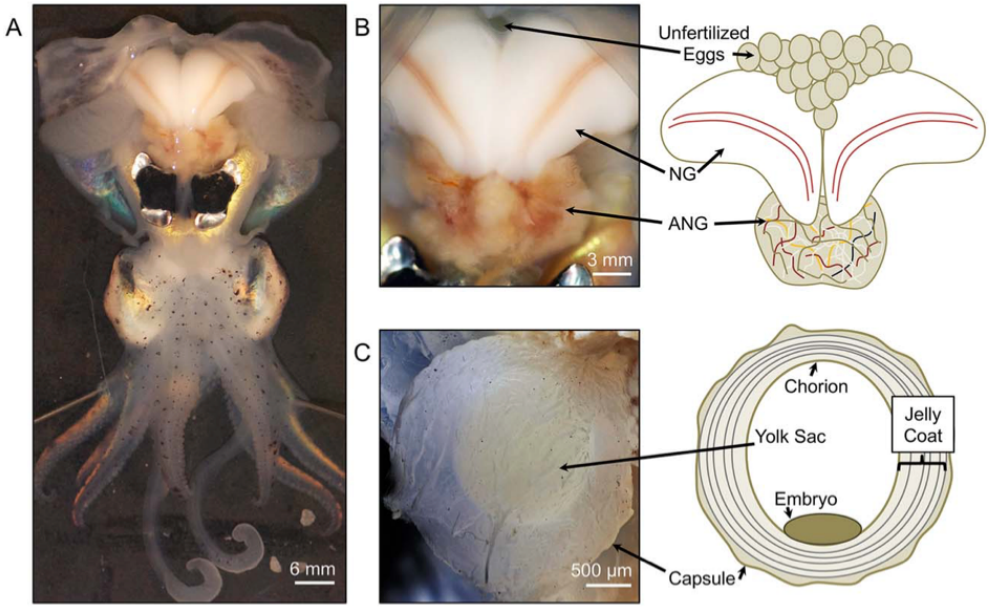


Fig. 1. Ventral dissection of a female *E. scolopes* (A), the female reproductive system (B) and an egg (C). Bacteria from the ANG are deposited into the JC layers that are secreted by the nidamental glands (NG). The embryo develops inside the central yolk sac, bounded by the chorion membrane. The JC surrounds the chorion and is encapsulated by an outer layer called the capsule which cements the egg to the rest of the clutch.

from corresponding individual females has not been reported.

Whether the ANG community is altered by maintaining mature animals in the laboratory is also unknown. Removing wild-caught animals from their native environments, especially when those environments are marine, and maintaining them in the laboratory can lead to changes in an animal's resident microbiota (Ford *et al.*, 1986; Kooperman *et al.*, 2007; Scott *et al.*, 2010; Devine *et al.*, 2012; Pratte *et al.*, 2015). While *E. scolopes* is easily maintained in the laboratory with little evident influence on fecundity, the effect of laboratory conditions on the ANG bacterial community is unclear.

E. scolopes lives in close contact with the microbial communities found in the environment, burrowing in the sediment during the day and actively swimming in the water column while hunting at night. Seawater flows constantly through the host's mantle cavity and this process is important in selecting *V. fischeri* for its light organ association (reviewed in Nyholm and McFall-Ngai, 2004). Microbial symbioses are transferred to the next generation either by direct maternal transfer of symbionts to offspring (vertical transmission), or by reacquiring symbionts from the environment (horizontal/environmental transmission; Bright and Bulgheresi, 2010). Juvenile cephalopods lack an ANG and are hypothesized to acquire their ANG symbionts from the environment during sexual development, despite the presence of those bacteria in the eggs (Kaufman *et al.* 1998, S. Nyholm pers. obs.). However, the microbial communities of the near-shore seawater and sediment in the natural habitat of *E. scolopes* remain poorly described. One goal of this research was to determine the seawater and sediment community composition of the bobtail squid's environment to understand whether bacteria associated with the ANG are present.

This research examines the variability of the ANG bacterial community of *E. scolopes* from Maunalua Bay, Oahu, Hawaii, and determines the core community of the ANG within this population. We analysed differences in bacterial composition of the ANG from wild and laboratory-maintained animals. Because the ANG association is thought to be environmentally transferred between generations, the bacterial communities of the seawater and sediment from Maunalua Bay were also examined. Finally, bacterial communities from the host's eggs were compared to ANGs, and the stability of the community during embryogenesis was characterized.

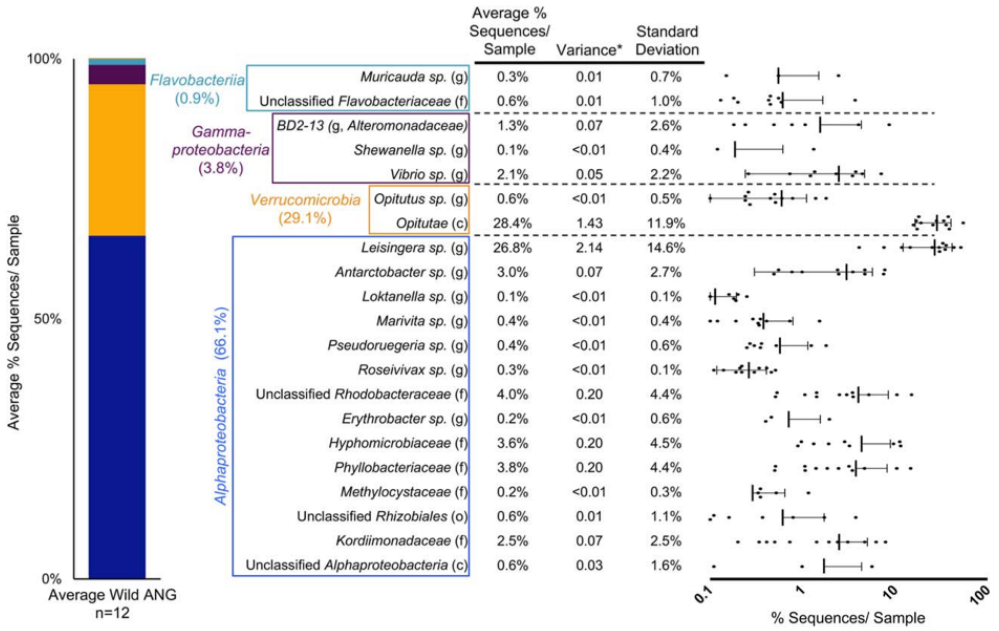


Fig. 2. The average symbiont population in the ANG of mature, wild-caught animals was composed of four bacterial classes. The percentage of sequences per sample for each taxon did not vary widely indicating a consistent community composition throughout the population of *E. scolopes*. Taxa present in more than one sample, and at greater than 0.1% of the average community, were included. Other taxa made up 0.15% of the community and included *Betaproteobacteria*, *Deltaproteobacteria* and *Sphingobacteriia* sequences. Taxa presented at the finest level obtained, c – class; o – order; f – family; g – genus. Mean % sequences/sample represented by thick bars, standard deviation represented by thin bars. Scatter plot is presented on a log scale to demonstrate variation for taxa present at lower average abundances. *Variance units are $\%^2$.

Results

High-throughput Illumina sequencing of the 16S rRNA V4 gene region revealed the phylogenetic diversity and variability of the ANG and JC bacterial communities across space and time. An examination of ANGs collected from wild mature bobtail squid ($n = 12$) showed that the average community was composed of four main bacterial groups: 66.1% ± 11.1% *Alphaproteobacteria*, 29.1% ± 11.8% *Verrucomicrobia*, 3.8% ± 3.6% *Gammaproteobacteria* and 0.9% ± 1.4% *Flavobacteriia* (Fig. 2). For all identified taxa the variance was low, indicating little individual differences in bacterial taxa between hosts from this population.

To determine whether bobtail squid maintained in the lab for an extended period had an altered ANG community, we compared lab-maintained animals to wild animals. ANGs from laboratory-maintained hosts ($n = 17$) had a similar composition to those from wild bobtail squid ($n = 12$, Fig. 3A). Operational taxonomic units (OTUs) identified as most closely related to *Betaproteobacteria* were only found in the ANGs of two of the lab-maintained animals, with one outlier at a comparatively higher relative abundance (2.4% ± 9.7%). ANGs from lab-maintained and wild *E. scolopes* clustered together via beta-diversity metrics (Fig. 3B), and one-way ANOSIM did not reveal significant dissimilarity between the groups ($R = -0.04$, $p = 0.79$), indicating that the bacterial consortium was stable when hosts were maintained in aquaria over their lifetime, usually a period of several months. When R is closer to zero in an ANOSIM analysis the similarity of the samples within a group is the same as the similarity between groups. Furthermore, animals collected over a period of seven years and different seasons had similar bacterial taxa, suggesting that the ANG community is stable over time and across generations.

The average JC community ($n = 35$) included fewer OTUs most closely related to the *Opitutae* class (*Verrucomicrobia*) than the average ANG community ($n = 29$, 8.0% vs. 25.0%), and more *Alphaproteobacteria* (71.3% vs. 65.0%, Fig. 4A). However, JC samples clustered with the

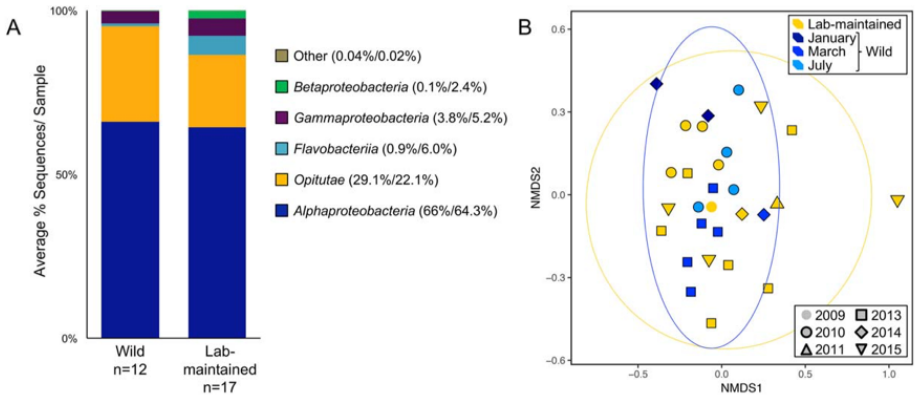


Fig. 3. Wild ($n = 12$) and lab-maintained ($n = 17$) ANGs had similar bacterial community compositions, both in the most abundant bacterial classes (A) and in beta-diversity (B). Bray Curtis NMDS plot of the two types of communities shows overlap of the sample types indicating that the community composition is similar. Shades of blue indicate month of collection for wild *E. scolopes*, while shape indicates year of collection. Community compositions were similar despite collection during different seasons and years. Ellipses indicate 95% confidence intervals of lab-maintained and wild groups. Taxa present in more than one sample, and at greater than 0.1% of the average community, were included (A). Other taxa made up 0.02% of the lab-maintained community and included *Deltaproteobacteria* and *Sphingobacteriia* sequences.

ANGs from wild and lab-maintained animals using beta-diversity metrics and one-way ANOSIM showed only low levels of dissimilarity between the groups ($R = 0.15$, $p = 0.001$), indicating that the bacterial consortium found in the JCs reflected that found in the ANG (Fig. 4B). While the spread of the JC samples was greater than that of the ANG samples which clustered closely together (Fig. 4B), the JCs and ANGs clustered more closely with each other than with environmental samples (Fig. 5A). JC samples generally clustered closer to the ANG of the female that produced those eggs than to other ANGs ($n = 5$), demonstrating that the JC community may reflect low levels of individual ANG variation (Fig. 4C, Supporting Information Fig. S1). JCs taken from various points in embryogenesis showed no clear clustering by embryonic stage. Clustering reflected the female that produced the eggs, but within each cluster the early- (day 0–2) and mid-stage (day 10–12) communities tended to group closer together and apart from the late-stage (day 17–24) community (Fig. 4D). Overall, this pattern indicates that the JC community is stable in terms of relative bacterial community composition throughout much of embryogenesis.

Eggs were also examined using TEM to determine whether the JC changed between early and late embryogenesis, and whether any patterns of bacterial distribution could be detected. During early embryogenesis, single bacterial cells were scattered throughout the JC layers, with no particular pattern in terms of cell morphology or distribution among inner vs. outer layers of the JC (Fig. 6A). By late embryogenesis single cells and small microcolonies of morphologically similar bacteria (typically 3–4 cells) were observed throughout the JC layers. Again, no pattern of distribution was observed (Fig. 6B). At both stages of embryogenesis, cells were observed in the process of cell division (Fig. 6C/D). An electron-dense material appeared to divide the various JC layers from each other (Fig. 6E). The abundance of culturable bacteria (CFUs) in the JC increased from an average of 2.1×10^4 CFU/JC at early embryogenesis (day 0) to an average of 3.0×10^5 CFU/JC by late embryogenesis (day 19–24, $n = 5$ clutches).

The Maunalua Bay *E. scolopes* ANG core bacterial community (OTUs present in 90% of ANG samples) consisted of 52 OTUs from two bacterial phyla, the *Verrucomicrobia* and the *Proteobacteria*. The *Verrucomicrobia* core members were all from the *Opitutae* class (12 OTUs), and in four cases were further identified to the genus *Opitutus* (Table 1). The *Proteobacteria* core members consisted solely of *Alphaproteobacteria* from the *Kordiimonadales* and *Rhizobiales* orders, and, most abundantly, the *Rhodobacteraceae* family. The majority of these core OTUs were classified to the family level of *Rhodobacteraceae* (17 OTUs), but five genera, specifically *Leisingera*, *Loktanella*, *Marivita*, *Roseivivax*, and *Antarctobacter* (14 OTUs) were also identified. The core community represented on average 79.5% of the sequences recovered per ANG sample. However, the abundance of those core OTUs varied from animal to animal, and the remaining 20.5% of sequences present in the average ANG was also variable, although the majority of the remaining OTUs belonged to the same taxonomic groups discussed here.

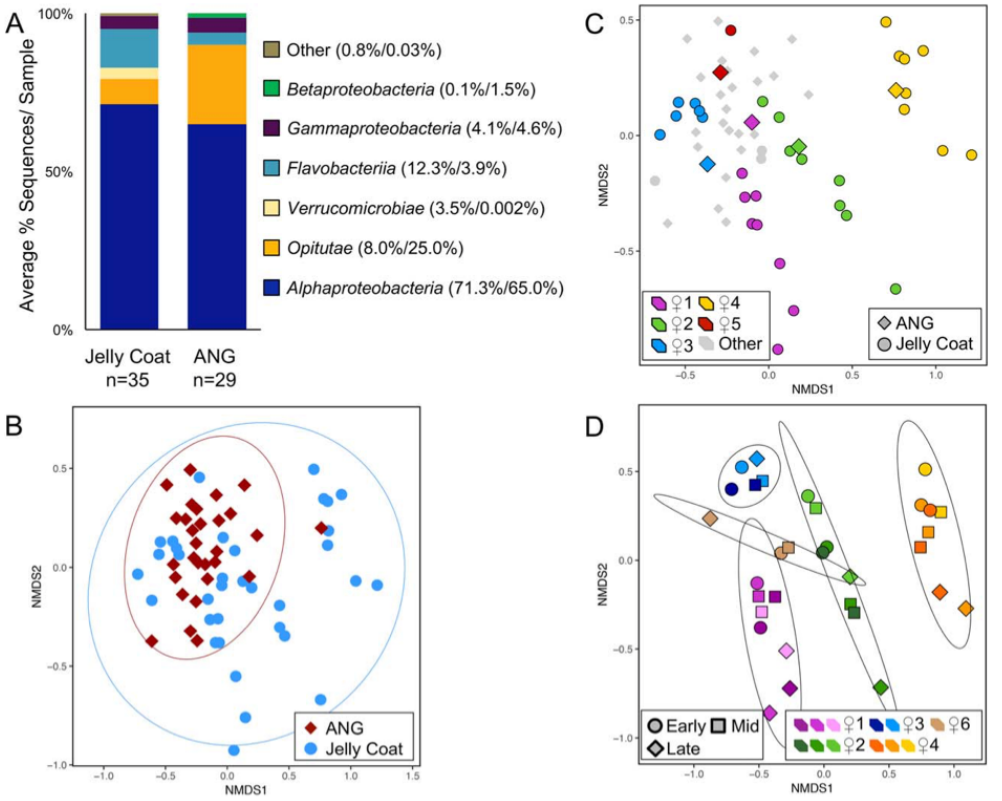
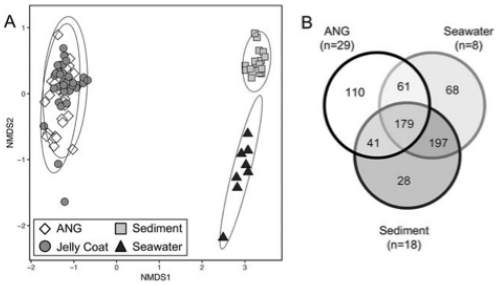


Fig. 4. The egg JC ($n = 35$) and ANG ($n = 29$) had similar bacterial community compositions, consisting of 4 main bacterial taxa (A). The 'other' component included taxa present in more than one sample, and at less than 0.5% of the average community composition. Other taxa made up 0.8% of the JC community and included unclassified *Bacteria* and *Proteobacteria*, *Clostridia*, *Deltaproteobacteria*, *Planctomycetia* and *Acidimicrobiia*. Bray Curtis NMDS analysis showed that the ANG and JC communities overlap (B). The same analysis demonstrated that JCs cluster with the ANG from the female that produced those eggs (C), and that JCs cluster by the individual female (colour), but not by stage of embryogenesis (D; shape, early = day 0–2, mid = day 10–12, late = day 17–24). JCs labelled as 'other' (C) are samples for which no matching ANG was analysed, or ANGs for which no matching JCs were obtained, and are provided for context. Ellipses indicate 95% confidence intervals (B/D).

The sediment community (n = 18) was similar throughout the sites sampled, and contained sequences belonging to 37 classes of bacteria and archaea (Supporting Information Fig. S2, Table S1). The seawater community ($n = 8$) was also similar between samples and contained sequences from 22 classes of bacteria and archaea (Supporting Information Fig. S3, Table S2). Both the sediment and seawater communities were more diverse than the ANG and JC communities, both in richness/evenness metrics (H' and E_H), and phylogenetically (PD, Fig. 5D). Beta-diversity metrics showed distinct clustering of the seawater and sediment samples apart from the ANG/JC samples, and one-way ANOSIM revealed significant dissimilarity between the environmental samples and bobtail squid-associated samples ($R = 1.0$, $p = 0.001$), indicating that the overall community composition of the three sample types was different (Fig. 5A). However, a substantial overlap of the OTUs present in the average ANG with those found in the environment was noted (Fig. 5B and C). Seventy-two percent of the 391 total OTUs recovered from all ANG samples were found in a seawater or sediment sample, or both. The OTUs unique to the ANG represented 5.5% of the average ANG sequences, and only 1.7% of the average core ANG sequences. These results suggest that the majority of the ANG community is also present in the environment.



C

	# OTUs	% of Average ANG Sequences	% of Average ANG Core Sequences
ANG/Sediment/Seawater	179	87.08%	75.11%
ANG/Sediment	41	2.26%	1.47%
ANG/Seawater	61	5.14%	1.24%
Unique to ANG	110	5.51%	1.65%
Total	391	100.00%	79.47%

D

	n	Shannon Index (H')	Shannon Equitability (E_H)	Phylogenetic Diversity (PD)
ANG	29	2.38 ± 0.38	0.51 ± 0.07	11.26 ± 2.32
Jelly coat	35	2.15 ± 0.76	0.47 ± 0.14	10.74 ± 2.63
Seawater	8	2.92 ± 0.36	0.60 ± 0.05	22.09 ± 3.71
Sediment	18	4.41 ± 0.19	0.82 ± 0.03	32.35 ± 2.58

Fig. 5. Comparison of the bacterial communities found in the Maunalua Bay seawater, sediment, JC, and ANG. The overall community composition of the sediment, seawater, and ANG/JC were distinct when compared via Bray Curtis beta-diversity analysis (A). However, the ANG community had substantial overlap with the seawater and sediment communities in terms of which OTUs were present (B) and in the percentage of the average ANG community those OTUs represented (C). Shannon index and equitability metrics indicated that the ANG and JC communities were less diverse than the seawater and sediment communities, and the same was true for phylogenetic diversity (D). Ellipses represent 95% confidence intervals (A).

Sequencing of the laboratory aquaria seawater and substrate revealed a subset of the natural bobtail squid environmental community (Supporting Information Fig. S4). While many of the bacterial classes present in the wild were also present in the lab, the relative abundances of those classes varied widely. The overlap of the ANG community with the lab environment was less than the overlap seen with the natural environment.

Discussion

The cephalopod ANG-bacteria association is widely distributed, but no study to date has examined the bacterial consortia of ANGs and eggs collected from individuals in the same environment. In this study, a core ANG community from *E. scolopes* was determined and found to be consistent and conserved in multiple mature female bobtail squid collected from Maunalua Bay, Oahu, HI. ANG communities were also stable when maintained in the lab over several months, making this symbiosis tractable for study in the laboratory. Furthermore, the OTUs that make up the ANG community were detected in the local environment, supporting the hypothesis of environmental symbiont transmission. Finally, the bacterial community of the egg JC reflected that of the associated ANG, and was stable throughout the duration of embryogenesis.

The ANG bacterial consortium was consistent and stable across individuals from this single population. For all taxa found in the ANG, the relative abundances of the bacterial community members did not vary substantially between individuals, indicating a stable community (Fig. 2). The core bacterial ANG community was dominated by two conserved members, the *Opitutae* (*Verrucomicrobia*) and *Rhodobacteraceae* (*Alphaproteobaceria*, Table 1), consistent with a previous study from *E. scolopes* (Collins *et al.*, 2012). Prior research and culturing efforts demonstrated that most of the *Rhodobacteraceae* found in the ANG belong to *Leisingera* sp. (Collins *et al.*, 2012, 2015), and while the 16S rRNA gene V4 region does not provide enough resolution to consistently resolve genera within the *Rhodobacteraceae* family, our results confirm that a majority of the ANG *Rhodobacteraceae* OTUs belong to *Leisingera* sp. (Fig. 2, Table 1). Prior research demonstrated that many of the *Rhodobacteraceae* isolates from the ANG are very similar to each other at the genome level (Collins *et al.*, 2015). Members of this group, commonly known as roseobacters, can be free-living or symbiotic (Collins *et al.*, 2015), and frequently produce pigments in culture, potentially accounting for the bright colouration of the tubules that make up the ANG (Fig. 1, Collins *et al.*, 2012; Gromek *et al.*, 2016). In the cuttlefish, *Sepia officinalis*, ANG pigmentation has been linked to the bacterial component of the organ (van den Branden *et al.*, 1979; 1980; Richard *et al.*, 1979). In this study, the *Rhodobacteraceae* accounted for 14.5% ± 5.12% of the average water column community and 3.2% ± 1.6% of the average sediment community in Maunalua Bay and thus are also a significant free-living component of the bobtail squid's natural habitat.

The second most dominant group of bacteria in the ANG was the *Opitutae* class of *Verrucomicrobia*. This group of bacteria is intriguing as it has only recently been shown to be involved in symbiotic associations (Vandekerckhovem *et al.*, 2000; Petroni *et al.*, 2000; Romero-Perez *et al.*, 2011). Few examples of symbiotic *Verrucomicrobia* are well described, but a closely related symbiotic verrucomicrobium has been described in a ciliate (Petroni *et al.*, 2000), and other *Verrucomicrobia* have been found in the human and bovine GI tracts (Romero-Perez *et al.*, 2011; Lozupone *et al.*, 2012). *Opitutae* were also found in both the sediment and seawater samples, but at very low

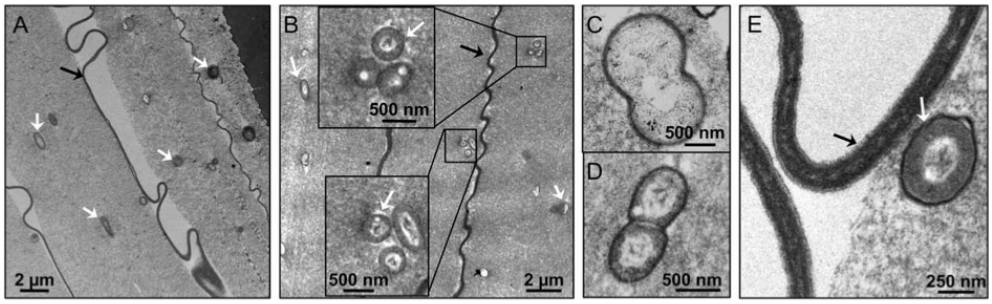


Fig. 6. Transmission electron micrographs of *E. scolopes* egg JCs collected at day 0 (A) or day 21 (B) of embryogenesis. At day 0 bacteria occurred as single cells throughout the various layers of JC (A), while at day 21 small microcolonies of bacteria of similar morphologies were common (B, see insets). Bacterial cells in the process of dividing were observed at both days 0 (C) and 21 (D). Layers of JC are separated by an electron-dense material composed of two layers (E). White arrows indicate bacterial cells and black arrows indicate membrane-like structures (A, B, E).

average relative abundances (0.3% ± 0.3% and 0.02% ± 0.01% respectively).

Both the *Rhodobacteraceae* and *Opitutae* are greatly enriched in the ANG community compared to both the sediment and water column. A similar enrichment can be seen for the *E. scolopes* light organ symbiont, where *V. fischeri* is abundant in the host but present only at low levels in the environment (Lee and Ruby, 1994, 1992). Sponge symbionts are also found at very low abundances in surrounding seawater and sediment environments, leading to the hypothesis that these rare microbes in the environment could serve as a 'seed bank' for colonization (Schmitt *et al.*, 2012; Thomas *et al.*, 2016). Future research will examine whether ANG bacteria are present at higher levels in local Hawaiian habitats with and without the host.

The 52 OTUs found in the core community represented 80% of the sequences present in the average ANG, providing further evidence of the stability and consistency of the ANG bacterial community. The animals included in this study were collected over the course of seven years, and no seasonal or yearly pattern was noted (Fig. 3). Because the lifespan of *E. scolopes* is predicted to be less than one year, the sampling in this study also represents multiple generations of bobtail squid from the same habitat. Such stability in a complex marine symbiosis appears uncommon: sponges and corals often have widely variable symbiont communities, and both microbiomes can contain thousands of OTUs (Ainsworth *et al.*, 2015; Thomas *et al.*, 2016). However, host-symbiont stability is a hallmark of other associations, including the binary symbioses of squid-vibrio associations or siboglinid tubeworms and sulfide-oxidizing bacteria (Dubilier *et al.*, 2008; McFall-Ngai, 2014).

Bringing animals into captivity and maintaining them in the laboratory often results in changes in their microbiota (Ford *et al.*, 1986; Scott *et al.*, 2010; Devine *et al.*, 2012). For example, the viable number of bacterial cells present on the mantle tissue of lab-maintained western Atlantic

Table 1. Core ANG bacterial community of *Euprymna scolopes* from Maunalua Bay. OTUs present at 97% identity level in 90% of 29 sampled bobtail squid are shown. OTUs in the core represent 79.5% of sequences present per average sample.

Phylum	Class	Order	Family	Genus	# OTUs	Average Abundance
		Kordiimonadales	*Kordiimonadaceae*		7	1.34%
		Rhizobiales	Unclassified *Rhizobiales*		1	0.05%
			Phyllobacteriaceae		1	3.01%
				Unclassified *Rhodobacteraceae*	17	15.56%
Proteobacteria	*Alphaproteobacteria*			*Leisingera sp.*	7	29.30%
		Rhodobacterales	*Rhodobacteraceae*	*Loktanella sp.*	1	0.08%
				Marivita sp.	1	0.34%
				Roseivivax sp.	1	0.14%
				Antarctobacter sp.	4	5.51%
		Unclassified *Opitutae*			8	23.18%
Verrucomicrobia	*Opitutae*	*Opitutales*	*Opitutaceae*	*Opitutus sp.*	4	0.95%
				Total	52	79.47%

brief squid, *Lolliguncula brevis*, increased ten-fold, a proliferation attributed to *Vibrio* sp. (Ford *et al.*, 1986). Studies examining corals, *Siderastrea siderea* and *Fungia granulosa*, the sea slug, *Elysia chlorotica*, and leaf-cutter ant gardens also found shifts in microbial communities when either aquatic or terrestrial animals were maintained in a laboratory (Kooperman *et al.*, 2007; Scott *et al.*, 2010; Devine *et al.*, 2012; Pratte *et al.*, 2015). To understand whether the ANG bacterial community is altered by laboratory conditions, ANG diversity was examined over the life of the bobtail squid. No shift was found in ANGs from laboratory-maintained animals, which were indistinguishable from those of wild individuals (Fig. 3). Such stability is not unprecedented, the gut microbial community of the Sonoran Desert turtle ant, *Cephalotes rohweri*, was unaffected by laboratory-maintenance (Lanan *et al.*, 2016), while the presence of the endosymbiont of the olive fruit fly, *Bactrocera oleae*, was also undisturbed by captivity under certain conditions (Estes *et al.*, 2012). The epithelia-associated microbiota of two species of *Hydra* maintains the species-specificity found in wild populations despite laboratory culture for over 30 years (Fraune and Bosch, 2007). *E. scolopes* has been used as a model system for studying host-microbe associations in part because animals are easily maintained and bred in the laboratory (Arnold *et al.*, 1972; Hanlon *et al.*, 1997; Nyholm and McFall-Ngai, 2004; Koch *et al.*, 2013). The stability of the ANG bacterial community suggests that this association may be studied intact over the adult life of the host under laboratory conditions, although whether the community changes over multiple reared generations has yet to be tested.

In certain cephalopods, the transfer of bacterial consortia from the ANG to eggs is hypothesized to aid in the protection of developing embryos, possibly from predation, pathogens, and/or biofouling (Biggs and Epel, 1991). Several members of the community have been shown to produce a number of secondary metabolites, some of which are able to inhibit certain marine vibrios (Collins *et al.*, 2015; Gromek *et al.*, 2016). A previous FISH analysis of eggs from *E. scolopes* demonstrated that JCs contain some of the same bacterial groups as those found in the ANG (Collins *et al.*, 2012). Despite differences in the relative abundances of the bacterial community between the JC and ANG of *E. scolopes*, we show that the JC community contains bacteria found in the ANG, and the bacteria in eggs produced by a given female group with that female's ANG (Fig. 4C, Supporting Information Fig. S1), providing evidence that bacteria from the ANG are deposited into the JC. The clustering of JCs by the associated female also accounts for the wider spread of the JC samples, which radiated outward from the ANG samples in the beta diversity analysis (Fig. 4C, Supporting Information Fig. S1). Since the ANG and JC of the squid *Doryteuthis pealeii* also have similar bacterial communities (Barbieri *et al.*, 2001), this deposition of ANG bacteria to eggs is likely to occur with other cephalopods.

If the bacteria in the JC play a defensive role for developing eggs, then the community should be conserved over the course of embryogenesis to maintain any protective effect. Once a female deposits her clutch, the eggs are potentially susceptible to fouling by microorganisms present in the seawater. The community composition of the JC remained stable despite the exposure of eggs to the environment for three weeks under laboratory conditions (Fig. 4D). The JC samples clustered by the female that produced the eggs and not by the stage of embryogenesis, although within those clusters the community seemed more similar at early- and mid-stages of embryogenesis. The differences in the late-stage communities were attributed to an occasional increase in *Flavobacteriia* (data not shown) but additional research is needed to confirm this observation.

While the overall community composition was not affected by embryonic stage, the bacteria in the JC did appear to be metabolically active. Microcolonies were detected during late embryogenesis along with bacterial cells in the process of cell division (Fig. 6). Cell division apparently occurred at a fairly slow rate given the small number of cells present in the microcolonies after 21 days. Culture-dependent estimates of bacterial abundance showed an average increase of an order of magnitude over the course of embryogenesis. These efforts to quantify the bacterial abundance in the JCs are an underestimation due both to an inability to culture the *Verrucomicrobia* contingent of the community, and difficulties in completely homogenizing the JC to ensure a uniform distribution prior to plating. Given these technical challenges, the overall measured abundance of the JC bacteria is a conservative estimate and the actual numbers are likely $>2 \times 10^4$ CFU/JC, and $>3 \times 10^5$ CFU/JC in early- and late-stage eggs respectively.

Transmission electron microscopy of eggs revealed the presence of a previously undescribed component of the JC, an electron-dense dual-layered structure that strongly resembles a membrane (Fig. 6E). This structure may be involved in maintaining the configuration of the egg capsule. Similar structures are visible in published images of other cephalopod eggs, including *D. pealeii*, *Rossia macrosoma*, *Loligo forbesi*, *D. opalescens*, and *Sepia officinalis*, although the structure is rarely commented upon (Biggs and Epel, 1991; Lum-Kong, 1992; Boletzky, 1998; Barbieri *et al.*, 2001; Cornet *et al.*, 2015). No segregation of the bacterial community based on morphological characteristics was observed in individual JC layers.

Despite the presence of ANG bacteria in the eggs, the ANG symbiosis is hypothesized to be environmentally transmitted. *E. scolopes* reaches sexual maturity in the laboratory in approximately 60 days (Hanlon *et al.*, 1997)

and the ANG develops between 1 and 1.5 months post-hatching (S. Nyholm, pers. obs.). While the ANG could be colonized by bacteria deposited in the juvenile at hatching and stored until ANG development begins, studies in other cephalopods suggest that ANG bacteria are likely environmentally transmitted (Kaufman *et al.*, 1998; Barbieri *et al.*, 2001) and that ANG development is correlated with this transmission (Kaufman *et al.*, 1998). Sepiolid (bobtail) squids are inherently benthic, spending time buried in sand and hunting in the water column. Colonization of the ANG may thus occur from bacteria in the seawater, substrate or both. The symbiosis itself could be a source of enrichment for the various symbionts in the environment as well, possibly after the juveniles hatch as the egg casings degrade. While it appears that the ANG does not experience the same daily venting as is seen in the light organ (reviewed in Nyholm and McFall-Ngai, 2004), the deposition of bacteria into the JC could result in the release of bacteria into the environment during egg laying. Comparative analysis of the ANG and environmental communities revealed substantial overlap in shared OTUs, with 94.5% of the average ANG sequences also found in the environment. The remaining OTUs may be rare members of the environment, accounting for the lack of detection to date.

Analysis of the microbial communities found in laboratory aquaria substrate and artificial seawater revealed less of an overlap in shared OTUs with the ANG community compared with the natural environment, (74.2% of the average ANG sequences accounted for, in comparison to 94.5%). While this analysis does not preclude the adult female as a source of enrichment for the ANG bacteria in her environment, it also does not provide strong evidence for the female seeding the environment, especially as no *Verrucomicrobia* were detected in either the laboratory artificial seawater or substrate. However, our laboratory conditions may have prevented the establishment of some ANG bacteria if released by females. Detected environmental bacteria in the laboratory could have been introduced during squid collection and transit.

The high overlap in the OTUs present in both the seawater and surface sediment (Fig. 5B) may have resulted from mixing of the communities during sampling, although the distinct clustering of the seawater community from that of the sediment, which takes into account OTU abundance, provides evidence that the two sampling methods resulted in distinct sample types (Fig. 5A). The seawater community was consistent across the sampling area, but varied from the Hawaii Ocean Time-Series (HOT) that characterized the bacterial and archaeal composition of the seawater at various depths approximately 100 km offshore of Oahu, HI (Supporting Information Fig. S3, Karner *et al.*, 2001; DeLong *et al.*, 2006; Brown *et al.*, 2009). Differences in the near-shore seawater community may be impacted by terrestrial runoff, anthropogenic activities, and the presence of certain algae (Smith *et al.*, 1999; Goeke *et al.*, 2010; Nogales *et al.*, 2011).

The presence of an ANG bacterial community is conserved throughout a diverse group of cephalopods (Buchner, 1965) but has only been examined via next-generation sequencing in *E. scolopes* (Collins *et al.*, 2012; this study). The conservation of a common bacterial community across many species, especially the *Alphaproteobacteria* (Grigioni *et al.*, 2000; Barbieri *et al.*, 2001; Pichon *et al.*, 2005), may reflect a conserved function of the ANG bacteria. Ongoing research in our laboratory is examining the putative role of these bacteria in egg protection. Differences in community composition between cephalopod species may be due to functional redundancy of the bacterial groups found, similar to what has been described in the mammalian gut (Ley *et al.*, 2006; Dethlefsen *et al.*, 2008), or could be a response to differing challenges found in environments where eggs develop. The conservation of the bacterial community in individual females across this population and the stability of the community for the duration of embryogenesis both support the hypothesis of a critical functional role in host development.

E. scolopes has served as a model organism for symbiosis research (McFall-Ngai, 2014). The ease of maintaining the host in the laboratory and the stability of the ANG consortium make this bobtail squid species an ideal candidate for studying cephalopod-ANG associations. Future efforts will also focus on examining the function and putative environmental transmission of this symbiosis. Developing a model for the comprehensive understanding of the establishment and maintenance of ANG bacteria will aid in our understanding of that community's function. Given the wealth of information obtained from decades of research about interactions with the light organ symbiont, studying the ANG symbiosis in *E. scolopes* may also provide insight into conserved and new mechanisms by which animals and symbiotic bacterial partners interact, both in the host and environment.

Experimental procedures

Animal collections

Female *E. scolopes* were collected from Maunalua Bay (21°26'3.36"N, 157°47'20.78"W), a sheltered sandflat on the island of Oahu, Hawaii, between March 2009 and August 2015. Bobtail squid were either sacrificed in Oahu (wild, $n = 12$) or were shipped to the University of Connecticut and maintained in the laboratory (lab-maintained, $n = 17$) for as long as four months (Supporting Information Fig. S5). Lab-maintained, mated females were kept in individual tanks, and egg clutches were moved within twelve hours of deposit to baskets in a separate tank, allowing for the tracking of eggs produced by an individual female. In one case, eggs were from a female that laid a clutch in tanks with flowing Hawaiian seawater (Kewalo Marine Laboratory, University of Hawaii,

Oahu, HI). Dissected tissues were surface-sterilized by washing first in 99% ethanol followed by filter-sterilized squid Ringer's solution (FSSR, Collins *et al.*, 2012).

DNA extraction: ANG. All sacrificed females were mature animals with a mantle length ≥20 mm, and had a fully developed reproductive system with eggs present in the mantle cavity. Prior to sacrifice, animals were anesthetized in 2% ethanol in filter-sterilized seawater (FSSW).

ANGs were homogenized in FSSR with a sterile plastic pestle. Differential centrifugation was used to separate bacterial cells from host tissue. The homogenized ANGs were centrifuged for five min at 100 X*g* to pellet the host tissue, then the supernatant containing the bacterial cells was removed and centrifuged for five min at 5000 X*g* to pellet the bacteria. DNA was extracted from samples using the DNeasy Blood and Tissue kit according to the manufacturer's protocol (Qiagen, Valencia, CA). The pelleted bacteria were combined with ATL buffer, Proteinase K, and zirconia beads (0.1 and 0.5 mm) and dissociated using a bead-beater for three min (Mini-Beadbeater-16, BioSpec Products, Bartlesville, OK). The solution was incubated for 30 min at 56°C, followed by bead-beating for an additional three min, and then incubated for 30 min at 56°C. Samples were centrifuged at room temperature for five min at 6000 X*g* to pellet the beads. DNA concentration was determined throughout using the Qubit® dsDNA High Sensitivity assay (ThermoFisher Scientific, Waltham, MA), and averaged 36.9 ng/μl ± 19.7 ng/μl (all samples >4 ng/μl, Supporting Information Fig. S6).

DNA extraction and bacterial quantification: egg JCs. Eggs were removed from a given clutch at various stages of embryogenesis after a clutch was deposited (early, day 0–2; mid, day 10–12; or late, day 17–24). Eggs were dissected using sterile forceps to remove the outer capsule and inner yolk sac, leaving only the JC.

DNA was extracted from 10 JCs/sample using the MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). JCs were first flash-frozen to −80°C for a minimum of 30 min. The provided Tissue and Cell Lysis buffer was prepared with Proteinase K to a final concentration of 0.833 μg/ml, and added to the frozen JCs with 0.1 and 0.5 mm zirconia beads. Samples were then subjected to bead-beating for five min followed by incubation at 65°C, with shaking overnight to allow the viscous JC material to break down, and subsequent bead-beating for five min. The manufacturer's protocol was then followed, repeating the protein precipitation step three times. DNA concentrations averaged 12.7 ng/μl ± 12.8 ng/μl (majority of samples >1 ng/μl, all samples >0.1 ng/μl, Supporting Information Fig. S6).

JC material from five eggs/sample was surface sterilized by washing first in 99% ethanol followed by FSSR. Samples were taken from early and late time points from the same clutches ($n = 5$). JCs were homogenized in FSSR using a sterile plastic pestle and then diluted prior to plating on triplicate seawater-tryptone plates (Lee and Ruby 1992). Colony counts were completed after three days of growth at 28°C.

Environmental DNA isolation. Sediment samples ($n = 18$) were collected via four transects at the site of animal collection, spanning approximately 600 m of the coast and extending 100–500 m from the shore towards the reef crest. Sterile tubes were used to collect the top three centimeters of sediment from four points on each transect. Samples were frozen at −80°C within one hour. Excess water was drained prior to processing to remove as much seawater from samples as possible. DNA extraction was completed on 250 mg of sediment via the DNeasy Blood and Tissue kit with bead-beating. DNA concentrations averaged 4.0 ng/μl ± 2.3 ng/μl (all samples >1 ng/μl, Supporting Information Fig. S6).

Seawater samples ($n = 8$) were collected from the points closest to shore and 250m from shore on each of the four transects described above. Samples were collected in sterile buckets and were transported back to the lab for immediate processing. Five liters of seawater from each collection point were filtered through 0.22 μm Whatman filters (GE Healthcare Life Sciences, Pittsburgh, PA) which were then frozen at −80°C. DNA was extracted using the PowerWater DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). DNA concentrations averaged 19.0 ng/μl ± 5.4 ng/μl (all samples >12 ng/μl, Supporting Information Fig. S6).

Substrate ($n = 3$) and artificial seawater ($n = 3$) samples were also collected from laboratory aquaria using the methods described above. Because aquaria water was circulated through particle and charcoal filters and subjected to UV sterilization, these samples resulted in low yields of total bacterial DNA. Laboratory substrate sample DNA concentrations averaged 0.8 ng/μl ± 0.1 ng/μl, while aquaria artificial seawater sample DNA concentrations averaged 0.3 ng/μl ± 0.2 ng/μl (Supporting Information Fig. S6). While use of low DNA concentration samples may increase the risk of contamination (Salter *et al.*, 2014), these samples were included for a point of comparison.

DNA amplification, sequencing, and analysis

Extracted DNA was amplified using barcoded primers developed by Caporaso *et al.*, (2012) for the V4 region of the 16S rRNA gene and sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) following established protocols (Nelson *et al.*, 2014; Benjamino and Graf, 2016). Some sample processing was performed by the UConn Microbial Analysis, Resources and Services facility.

Sequencing data were analysed following established protocols (Nelson *et al.*, 2014; Benjamino and Graf, 2016) using QIIME (Caporaso *et al.*, 2010). OTUs were assigned at the 97% identity level using Greengenes (2013-08 release, DeSantis *et al.*, 2006) and *de novo* methods. The dataset was rarified to 10,000 sequences per sample. A core community was determined as OTUs present in 90% of ANG samples. Alpha diversity was analysed in QIIME, and the log2 Shannon Index was converted to a natural log Shannon Index. NMDS plots of beta-diversity analyses using Bray-Curtis were created in *R* using the VEGAN package (Oksanen *et al.*, 2016), and community composition similarity was tested via ANOSIM in QIIME. Sequences were deposited in the European Nucleotide Archive (ENA) under the project ID PRJEB14655.

Transmission electron microscopy (TEM)

A freshly deposited *E. scolopes* clutch was maintained in aerated FSSW, which was changed daily. At 0 and 21 days post-

deposit, eggs were obtained from the clutch and the outer capsule was removed. Decapsulated eggs were prepared for TEM following established protocols (Collins *et al.*, 2012) with the following alterations. Eggs were fixed (2.5% gluteraldehyde/2% paraformaldehyde solution, Collins *et al.*, 2012) at room temperature for 1 h, placed at 4°C for 10 min, and then transferred to fresh fixative and stored at 4°C for up to 22 days. Post-fixation protocols were carried out on all treatments at the end of the experiments, but no anomalies were noted in the day 0 eggs, which were stored in fixative the longest. The yolk sac (Fig. 1) was pierced prior to osmication to allow for complete infiltration during the remaining steps. After ethanol dehydration, eggs were transferred to a transition fluid, 100% propylene oxide, for two washes of 15 min. Tissues were embedded in Spurr's resin and sectioned on a Leica UCT Ultramicrotome (Leica Microsystems, Buffalo Grove, IL) into 90 nm ultrathin sections. Samples were imaged on a Tecnai Biotwin transmission electron microscope (7–12 sections/sample, FEI, Hillsboro, OR).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Bray Curtis NMDS analysis showed that JCs (circles) cluster with the ANG (diamond) from the female (colour) that produced those eggs. Ellipses indicate 95% confidence intervals. This figure is another version of that shown in Figure 4C, but includes only the groups of ANGs/JCs. Female 5 from that analysis is left out here as it included only a single JC with the ANG, and a 95% confidence interval requires at least three data points.

Fig. S2. The average sediment sample contained 13 classes from seven bacterial/archaeal phyla. Taxa present at greater than 1% average abundance in more than one sample are included, and presented at the class level. Mean % sequences/sample represented by thick bars, standard deviation represented by thin bars. Scatter plot is presented on a log scale to demonstrate variation for taxa present at lower average abundances. The 'other' category includes taxa present at less than 1% average abundance: *Acidobacteria-6*, *Gemm-2*, *Gemm-4*, *Ellin6529*, *Opitutae, Nitrospira, Phyciphaerae, PRR-12*, *OM190*, *SAR202*, *Bacteroidi*a, *C6*, *Chloracidobacteria*, *VHS-B5-50*, *Fusobacteria, Gemm-1*, unclassified *Planctomycetes*, *Actinobacteria*, *Chlamydiia*, *Bacilli*, *Anaerolineae*, *Verrucomicrobiae*, *Synechococcophycideae*, and *Clostridia*. *Probably of eukaryotic macro- or microalgal origin.

Fig. S3. The average seawater sample contained six classes from four bacterial/archaeal phyla. Taxa present at greater than 1% average abundance in more than one sample are included and presented at the class level. Mean % sequences/sample represented by thick bars, standard deviation represented by thin bars. Scatter plot is presented on a log scale to demonstrate variation for taxa present at lower average abundances. The 'other' category includes taxa present at less than 1% average abundance: *Thaumarchaeota*, *Acidimicrobiia, Planctomycetia*, *Deltaproteobacteria*, *Fusobacteria*, *Oscillatoriophycidae*, *Betaproteobacteria*, *Epsilonproteobacteria*, *Opitutae*, *Ellin6529*, *Acidobacteria-6*, *Sphingobacteriia*, *Gemm-2*, *OM190*, *Gemm-4*, and *Sva-0725*. *Probably of eukaryotic macro- or microalgal origin.

Fig. S4. Laboratory artificial seawater (n=3) and substrate (n=3) contained many of the same taxa as the natural Hawaiian environment, but at different relative abundances (A, B, D). Taxa present at greater than 1% are included (A). The lab environment contained fewer OTUs that overlapped with the ANG community than the natural environment (C). Lab substrate exhibited similar levels of diversity as natural Hawaiian sediment, but the lab artificial seawater was more diverse than that from Hawaii (E). *Probably of eukaryotic macro- or microalgal origin.

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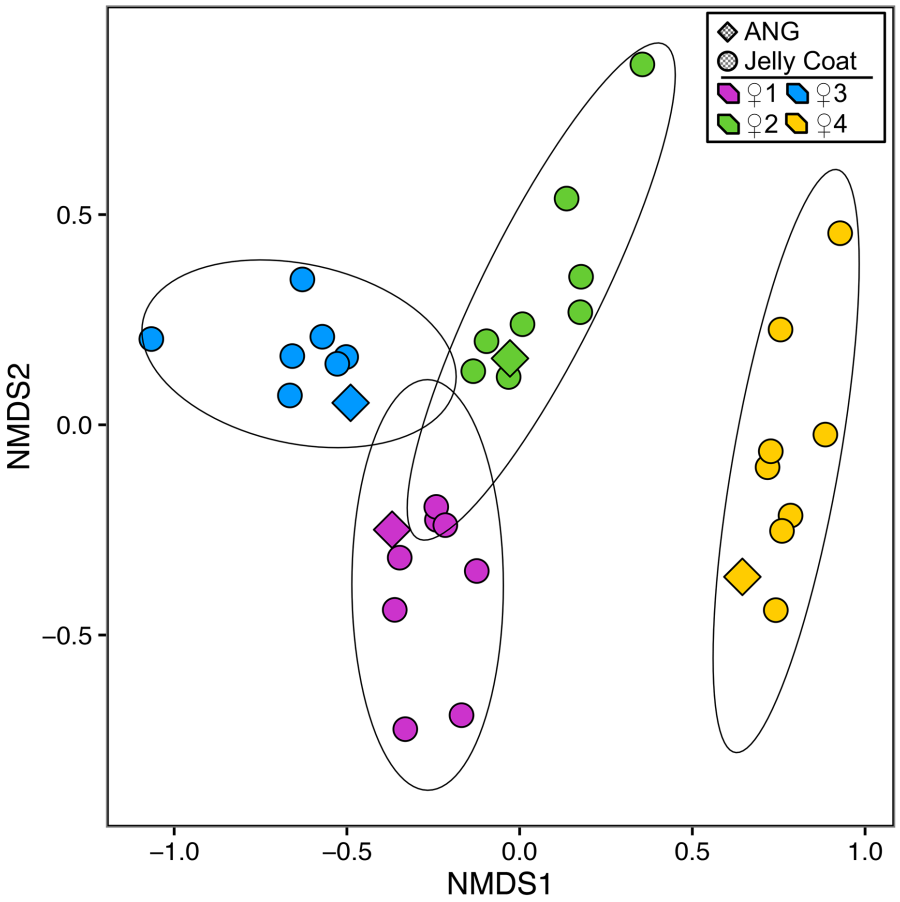
Table S1. Conserved bacterial community of the Maunalua Bay, Oahu, HI sediment. OTUs present at 97% identity level present in 90% of 18 sediment samples are shown. Conserved OTUs represent 87% of sequences present per average sample. *Probably of eukaryotic macro- or microalgal origin.

Table S2. Conserved bacterial community of the Maunalua Bay, Oahu, HI seawater. OTUs present at 97% identity level present in 85% of 8 water samples are shown. Conserved OTUs represent 98.4% of sequences present per average sample. *Probably of eukaryotic macro- or microalgal origin.



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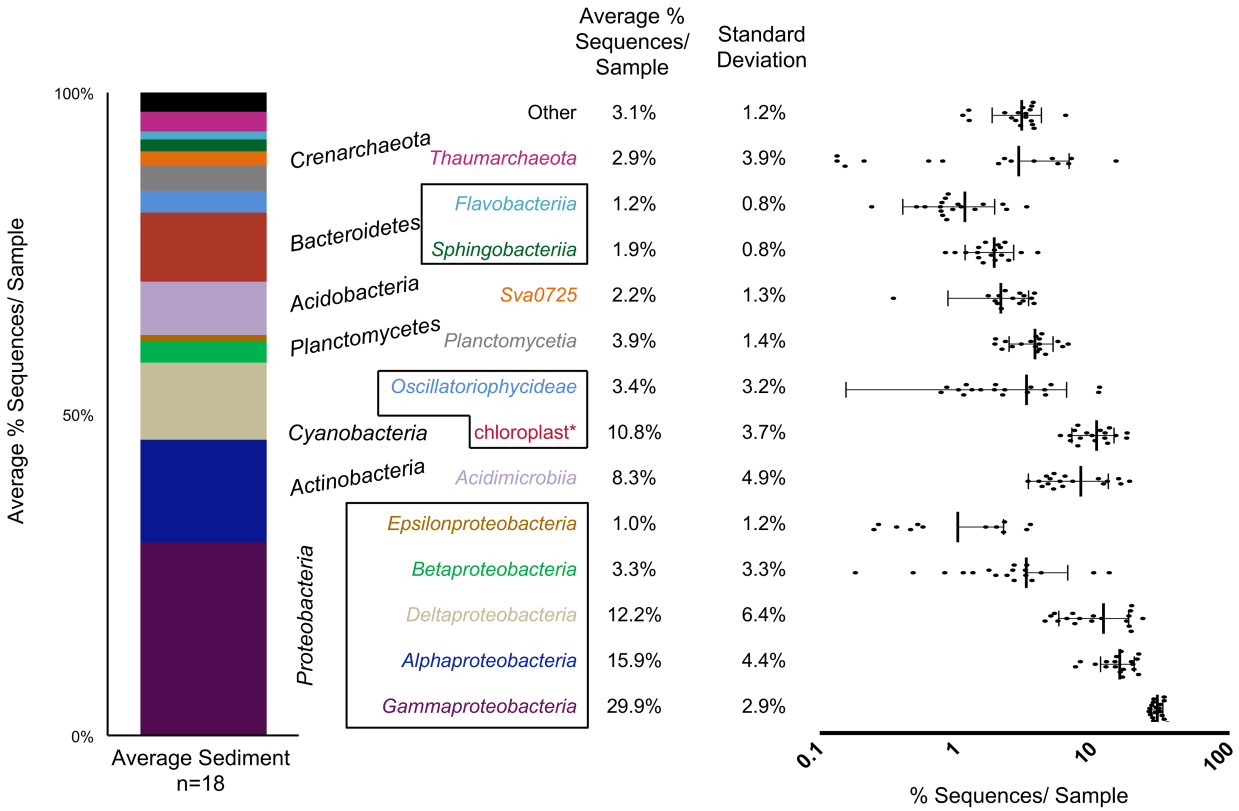

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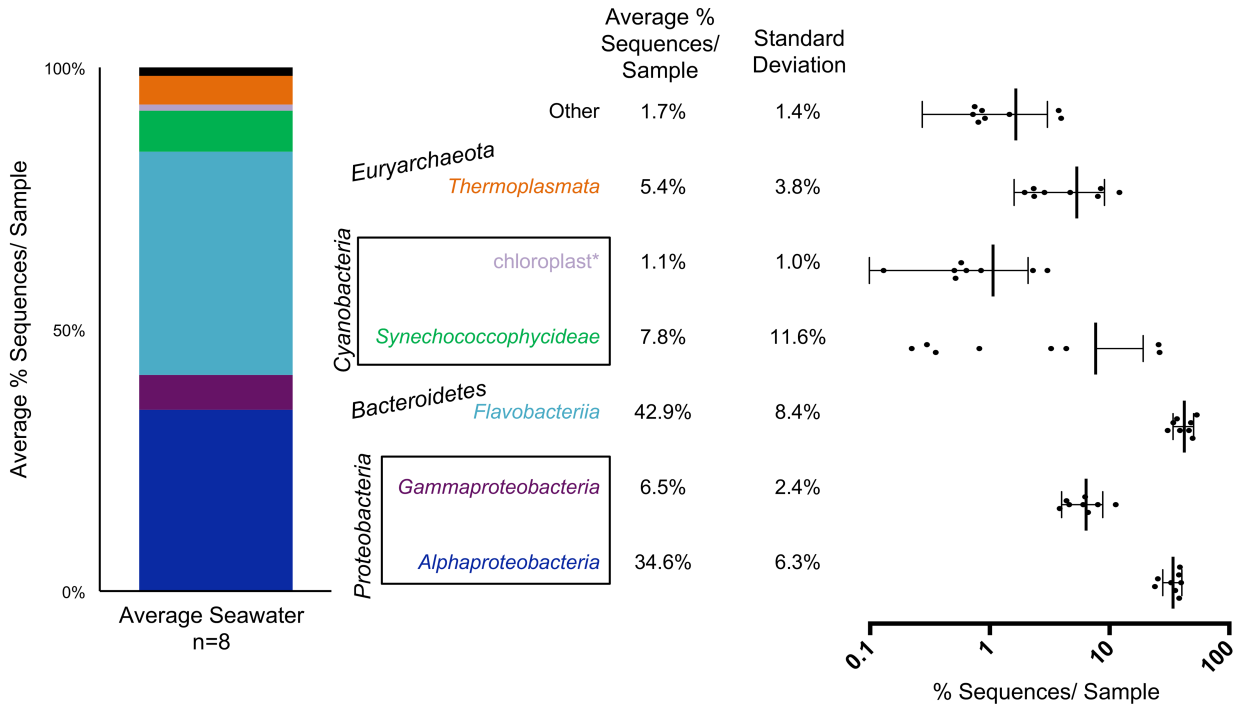

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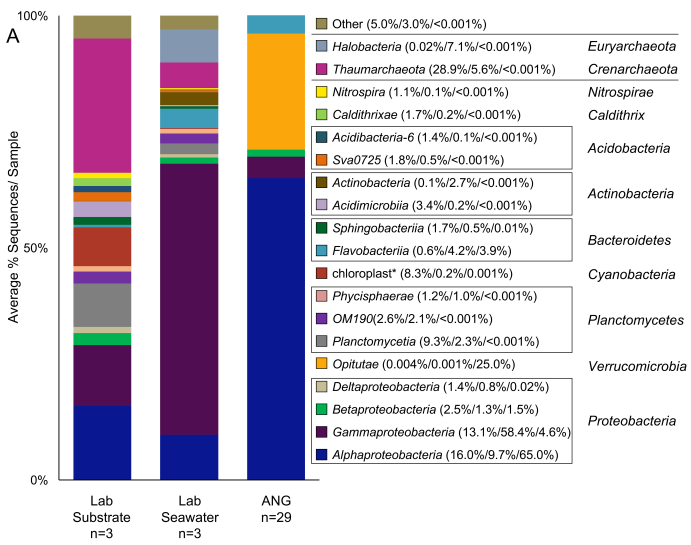
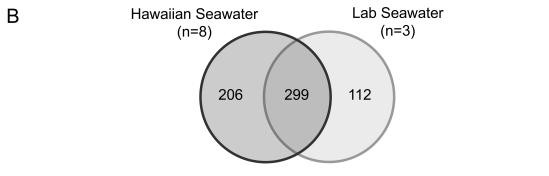
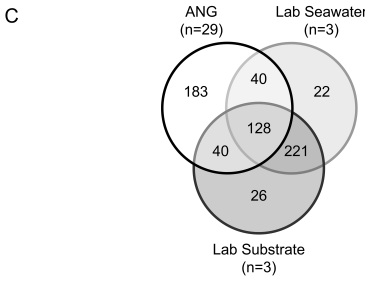




	# OTUs	% of Average Hawaiian Seawater Sequences	% of Average Lab Seawater Sequences
Hawaiian/Lab Seawater	299	41.69%	77.52%
Hawaiian Seawater Only	206	58.31%	-
Lab Seawater Only	112	-	22.48%



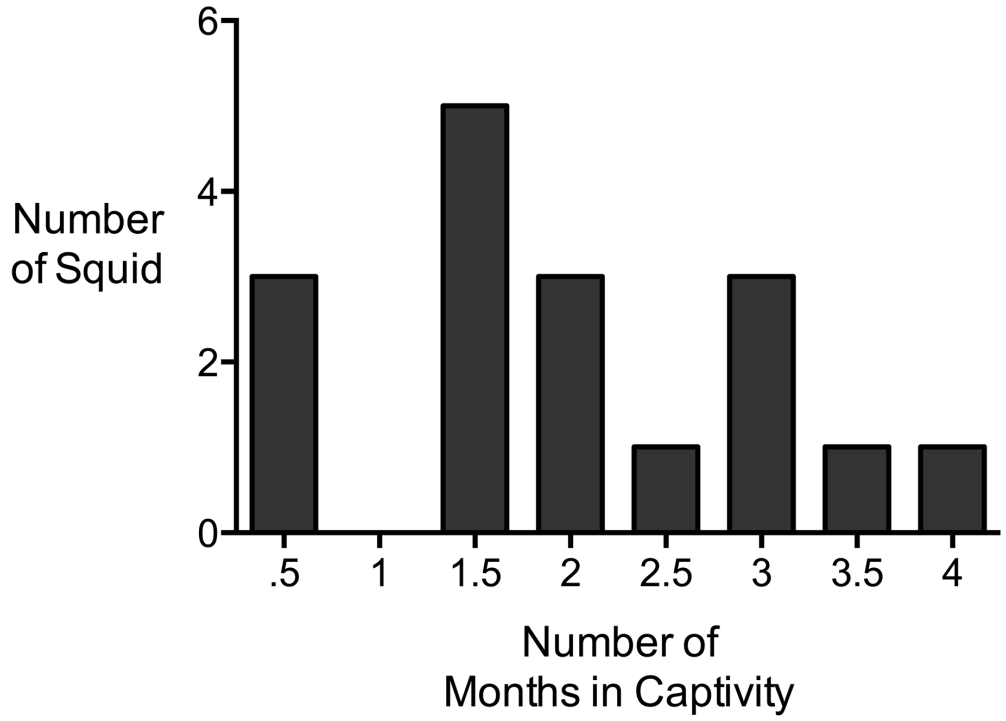
	# OTUs	% of Average ANG Sequences	% of Average ANG Core Sequences
ANG/Sediment/Seawater	128	64.63%	52.38%
ANG/Sediment	40	1.45%	0.14%
ANG/Seawater	40	8.10%	7.22%
Unique to ANG	183	25.82%	19.72%
Total	391	100.00%	79.47%

	# OTUs	% of Average Hawaiian Sediment Sequences	% of Average Lab Substrate Sequences
Hawaiian/Lab Sediment	299	65.48%	89.51%
Hawaiian Sediment Only	206	34.52%	-
Lab Sediment Only	112	-	10.49%

E

	n	Shannon Index (H')	Shannon Equitability (E_H)	Phylogenetic Diversity (PD)
Lab Substrate	3	4.34 ± 0.34	0.79 ± 0.06	36.34 ± 0.32
Lab Seawater	3	3.65 ± 0.71	0.68 ± 0.10	34.23 ± 8.45

Supplemental Figure SF4. Laboratory artificial seawater (n=3) and substrate (n=3) contained many of the same taxa as the natural Hawaiian environment, but at different relative abundances (A, B, D). Taxa present at greater than 1% are included (A). The lab environment contained fewer OTUs that overlapped with the ANG community than the natural environment (C). Lab substrate exhibited similar levels of diversity as natural Hawaiian sediment, but the lab artificial seawater was more diverse than that from Hawaii (E). *Probably of eukaryotic macro- or microalgal origin.



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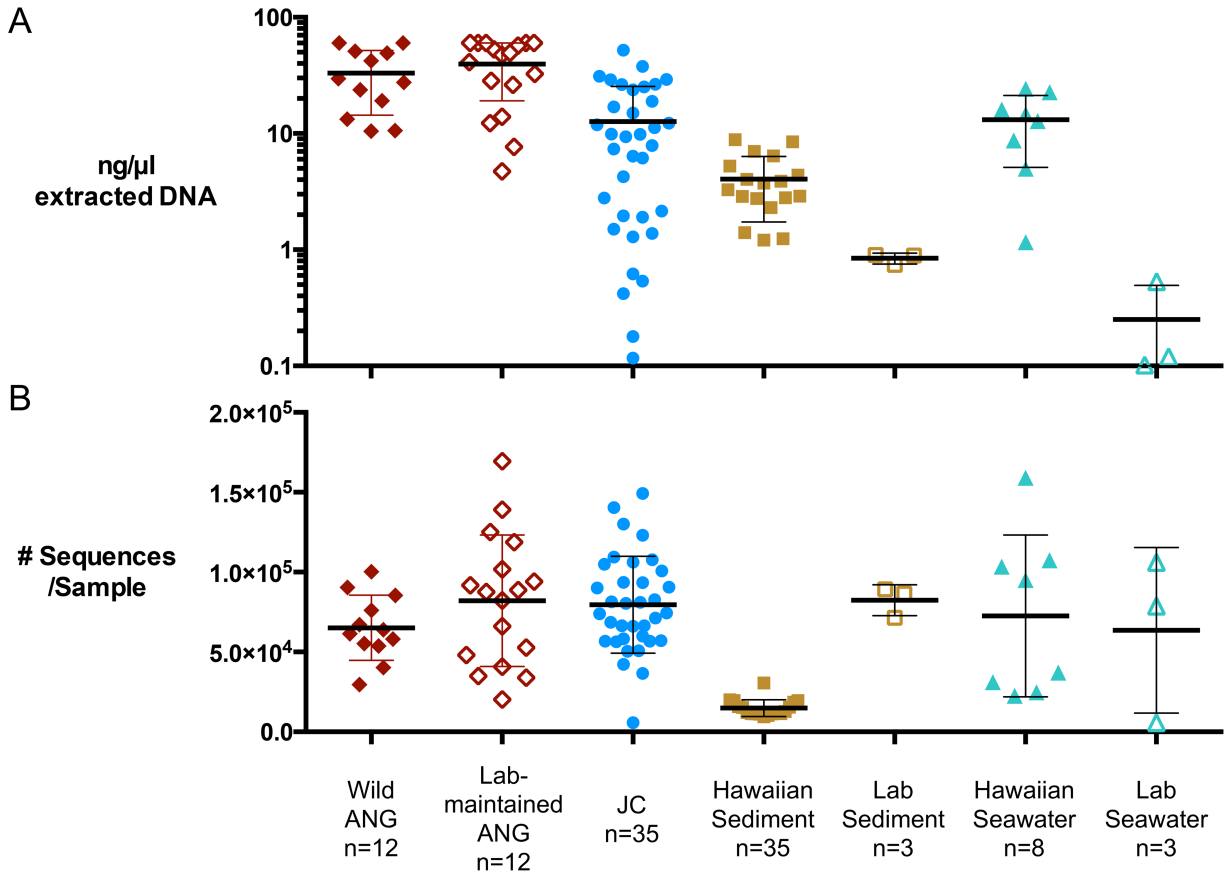

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Supplemental Table ST1. Conserved bacterial community of the Maunalua Bay, Oahu, HI sediment. OTUs present at 97% identity level present in 90% of 18 sediment samples are shown. Conserved OTUs represent 87% of sequences present per average sample. *Probably of eukaryotic macro- or microalgal origin.

Domain	Phylum	Class	Order	Family	Genus	# OTUs	Average Abundance
Archaea	*Crenarchaeota*	*Thaumarchaeota*	*Cenarchaeales*	*Cenarchaeaceae*	*Nitrosopumilus sp.*	1	0.49%
Bacteria	*Acidobacteria*	*Acidobacteria-6*	*BPC015*			2	0.55%
		Chloracidobacteria				1	0.04%
		Sva0725	*Sva0725*			3	1.88%
	Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	*C111*		4	3.93%
				JdFBGBact		3	0.79%
				koll13		2	2.01%
				wb1_P06		1	0.14%
				ZA3409c		2	0.61%
	Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	Unclassified *Flavobacteriaceae*	2	0.59%
					Lutimonas sp.	1	0.38%
		Sphingobacteriia	*Sphingobacteriales*	*Rhodothermaceae*		1	0.06%
				Chitinophagaceae		1	0.59%
				Ekhidnaceae		2	1.03%
	Chloroflexi	*Ellin6529*				1	0.26%
	Cyanobacteria	*chloroplast**	CAB-1			1	1.45%
			Stramenopiles			5	9.31%
		Oscillatoriophycideae	*Chroococcales*	Unclassified *Chroococcales*		3	1.39%
				Xenococcaceae	*Xenococcus sp.*	1	0.89%
	Gemmati-monadetes	*Gemm-2*				2	0.35%
		Gemm-4				2	0.80%
	Planctomycetes	*C6*	*d113*			1	0.04%
		Phycisphaerae	*Phycisphaerales*			1	0.09%
		Planctomycetia	*Pirellulales*	*Pirellulaceae*		14	3.50%

Domain	Phylum	Class	Order	Family	Genus	# OTUs	Average Abundance
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	Unclassified *Alphaproteobacteria*			1	0.16%
			Caulobacterales	*Caulobacteraceae*		1	0.11%
			Rhizobiales	*Bradyrhizobiaceae*	*Bradyrhizobium sp.*	1	0.61%
				Cohaesibacteraceae		1	0.34%
				Hyphomicrobiaceae		4	0.85%
				Rhodobiaceae		1	0.09%
			Rhodobacterales	*Hyphomonadaceae*		1	0.12%
				Rhodobacteraceae	Unclassified *Rhodobacteraceae*	7	2.42%
					Antarctobacter sp.	1	0.02%
					Octadecabacter sp.	1	0.22%
					Leisingera sp.	1	0.16%
			Rhodospirillales	Unclassified *Rhodospirillales*		1	0.83%
				Rhodospirillaceae	Unclassified *Rhodospirillaceae*	7	7.09%
					Inquilinus sp.	1	0.78%
		Betaproteobacteria	Unclassified *Betaproteobacteria*			1	0.08%
			Burkholderiales	*Comamonadaceae*		4	2.80%
				Oxalobacteraceae		1	0.33%
		Deltaproteobacteria	*Desulfobacterales*	*Desulfobulbaceae*		3	3.24%
			Myxococcales			2	2.30%
			NB1-j	Unclassified *NB1-j*		2	1.15%
				NB1-i		2	0.66%
			Syntrophobacterales	*Desulfobacteraceae*	*Desulfococcus sp.*	3	2.16%
					Desulfosarcina sp.	1	0.76%
		Epsilonproteobacteria	*Campylobacterales*	*Helicobacteraceae*		1	1.00%

Domain	Phylum	Class	Order	Family	Genus	# OTUs	Average Abundance
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	Unclassified *Gammaproteobacteria*			2	1.79%
			Alteromonadales	*OM60*	Unclassified *OM60*	3	1.04%
				Shewanellaceae	*Shewanella sp.*	1	1.27%
			Chromatiales	Unclassified *Chromatiales*		4	2.74%
			HTCC2188	*HTCC2089*	Unclassified *HTCC2089*	2	0.75%
			Oceanospirillales	*Halomonadaceae*	*Halomonas sp.*	2	1.76%
			Methylococcales	*Unclassified Methylococcales*		1	0.55%
				Methylococcaceae		5	2.69%
			Xanthomonadales	*Unclassified Xanthomonadales*		9	14.48%
				Sinobacteraceae		1	0.48%
	SBR1093	*VHS-B5-50*				1	0.04%
	Verrucomicrobia	*Opitutae*				1	0.05%
					Total	138	87.08%

Supplemental Table ST2. Conserved bacterial community of the Maunalua Bay, Oahu, HI seawater. OTUs present at 97% identity level present in 85% of 8 water samples are shown. Conserved OTUs represent 98.4% of sequences present per average sample. *Probably of eukaryotic macro- or microalgal origin.

Domain	Phylum	Class	Order	Family	Genus	# OTUs	Average Abundance
Archaea	*Crenarchaeota*	*Thaumarchaeota*	*Cenarchaeales*	*Cenarchaeaceae*	Unclassified *Cenarchaeaceae*	2	0.03%
					Cenarchaeum sp.	1	0.09%
					Nitrosopumilus sp.	3	0.28%
	Euryarchaeota	*Thermoplasmata*	*E2*	*Marine group II*		2	5.43%
Bacteria	*Actinobacteria*	*Acidimicrobiia*	*Acidimicrobiales*	*koll13*		1	0.01%
				OCS155		2	0.69%
	Bacteroidetes	*Flavobacteriia*	Unclassified *Flavobacteriia*			7	24.84%
			Flavobacteriales	*Flavobacteriaceae*	Unclassified *Flavobacteriaceae*	13	17.80%
					Lutimonas sp.	1	0.01%
					Bizonia sp.	1	0.01%
					Polaribacter sp.	1	0.01%
					Tenacibaculum sp.	2	0.09%
	Chloroflexi	*Ellin6529*				1	0.01%
	Cyanobacteria	*chloroplast**	*CAB-I*			1	0.01%
			Cryptophyta			1	0.11%
			Haptophyceae			1	0.13%
			Stramenopiles			8	0.84%
		Oscillatoriophycideae	*Chroococcales*	Unclassified *Chrococcales*		1	0.01%
				Xenococcaceae	*Xenococcus sp.*	1	0.01%
		Synechococcophycideae	*Pseudanabaenales*	*Pseudanabaenaceae*	*Halomicronema sp.*	1	0.03%
			Synechococcales	*Synechococcaceae*	*Prochlorococcus sp.*	3	7.45%
	Fusobacteria	*Fusobacteria*	*Fusobacteriales*	*Fusobacteriaceae*	*Propionigenium*	1	0.09%
	Gemmatimonadetes	*Gemm-2*				1	0.01%
	Planctomycetes	*Planctomycetia*	*Pirellulales*	*Pirellulaceae*		7	0.11%

Domain	Phylum	Class	Order	Family	Genus	# OTUs	Average Abundance
			Kiloniellales			1	0.47%
			Rhizobiales	Unclassified *Rhizobiales*		1	0.01%
				Hyphomicrobiaceae		2	1.79%
				Rhodobiaceae		1	0.02%
				Methylocystaceae		1	0.01%
		Alphaproteobacteria	*Rhodobacterales*	*Hyphomonadaceae*		1	0.02%
				Rhodobacteraceae	Unclassified *Rhodobacteraceae*	15	14.13%
					Leisingera sp.	3	0.17%
					Antarctobacter sp.	1	0.01%
					Octadecabacter sp.	1	0.01%
Bacteria	*Proteobacteria*				*Pseudoruegeria sp.*	1	0.02%
			Rhodospirillales	Unclassified *Rhodospirillales*		1	0.01%
				Rhodospirillaceeae		12	16.23%
			Rickettsiales	Unclassified *Rickettsiales*		3	1.02%
				AEGEAN_112		1	0.11%
				Pelagibacteraceae		2	0.12%
		Betaproteobacteria	*Methylophilales*	*Methylophilaceae*	*Methylotenera sp.*	1	0.03%
		Deltaproteobacteria	*Syntrophobacterales*	*Desulfobacteraceae*	*Desulfococcus sp.*	1	0.01%
			Desulfobacterales	*Desulfobulbaceae*		3	0.06%
			Myxococcales			1	0.01%
		Epsilonproteobacteria	*Campylobacterales*	*Helicobacteraceae*		1	0.02%

Domain	Phylum	Class	Order	Family	Genus	# OTUs	Average Abundance
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	Unclassified *Gammaproteobacteria*			3	0.03%
			Alteromonadales	*OM60*		4	2.88%
				Pseudoalteromonadaceae	*Pseudoalteromonas sp.*	2	0.04%
			Chromatiales			2	0.02%
			Oceanospirillales	*Litoricolaceae*	*Litoricola sp.*	1	1.83%
				Halomonadaceae	Candidatus Portiera	4	0.79%
				Oceanospirillaceae		1	0.03%
			HTCC2188	HTCC2089		2	0.02%
			Vibrionales	*Vibrionaceae*	*Photobacterium sp.*	1	0.01%
					Vibrio sp.	5	0.26%
			Xanthomonadales			5	0.08%
					Total	147	98.37%

Chapter 3

The complex community of bobtail squid eggs: diverse functionality with a common goal

Significant Contributions

Significant contributions to this chapter were made by other researchers. Dr. Sal Frasca, University of Connecticut, and Dr. Deanna Sutton, Fungal Testing Laboratory, San Antonio, TX, isolated and identified *F. keratoplasticum*, and provided an additional laboratory strain for extract testing. Dr. Kerry O'Donnell, National Center for Agricultural Utilization Research, Agricultural Research Service, Peoria, IL, completed MLST of the *F. keratoplasticum* isolates from CT and HI, and provided the phylogeny for Figure 4. Andrea Suria, University of Connecticut, isolated the ANG/JC strains used for organic extractions and antifungal assays. Samantha Gromek and Dr. Marcy Balunas, University of Connecticut, obtained the organic extracts and completed the antifungal assays, generating the data used for Figure 9.

Abstract

Many marine/aquatic organisms deposit their eggs in an environment where successful embryogenesis depends on minimizing biofouling and/or predation. The Hawaiian bobtail squid, *Euprymna scolopes*, harbors a diverse bacterial community within the accessory nidamental gland (ANG), a symbiotic organ associated with the female reproductive system. That bacterial community is added to the jelly coat (JC) of eggs prior to deposit on a substrate. Eggs treated with antibiotics over the course of embryogenesis developed a biofilm, primarily composed of the fungus *Fusarium keratoplasticum*, which led to the death of the embryos (9% hatch; n = 5 clutches). Transmission electron microscopy confirmed that both fungal hyphae and spores penetrated the JC of antibiotic-treated eggs as far as the chorion. Fungal challenge experiments on dissected eggs demonstrated that the egg JC containing the bacterial community is essential for egg defense from fungal bud cells (n=3 trials, 8-10 eggs/treatment). Finally, extracts from ANG/JC bacteria were also able to inhibit *F. keratoplasticum* and *Candida albicans in vitro*. Taken together, these data suggest that the ANG/JC bacteria protect developing embryos from biofouling. We propose a model for egg defense in *E. scolopes*, by which specific members of the JC bacterial community produce various secondary metabolites to prevent certain threats. The *E. scolopes* ANG bacterial consortium is shown to provide a tractable system to explore functional specificity in a marine symbiosis. Understanding the function of the ANG bacterial consortium may lead to novel anti-fouling/antimicrobial compounds and lend insight into the mechanisms by which marine invertebrates protect their eggs in the environment.

Introduction

Defensive symbioses, wherein antimicrobial compounds derived from beneficial microbes are used to inhibit other microorganisms, are found in a number of host-microbe

associations (Fraune et al. 2015, Gil-Turnes and Fenical 1992, Gil-Turnes et al. 1989). This phenomenon has been studied in terrestrial and insect associations, for example in a number of beetle species, termites, fungus-farming ants, and in the protection of beewolf larvae (reviewed in Flórez et al. 2015). Many organisms also lay their eggs in an environment where successful embryogenesis depends on minimizing fouling by microorganisms. Aquatic organisms are especially susceptible to fouling since their eggs are under constant exposure to high densities of microorganisms, for example, over 10^6 bacterial cells/ml and 10^3 fungal spores/ml in most coastal seawater (Goeke et al. 2010). Given that embryogenesis can often take weeks and biofilms can generally form in a matter of hours to days, mechanisms must be present to prevent microbial growth on externally laid eggs.

Many female cephalopods maintain a bacterial symbiotic community within their reproductive tract in an organ known as the accessory nidamental gland (ANG, Figure 1, Buchner 1965). This organ typically contains a community composed of some combination of *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia*, depending on the host species (Barbieri et al. 2001, Grigioni et al. 2000, Pichon et al. 2005, Collins et al. 2012, Kerwin and Nyholm 2017). These bacteria are added to the jelly coat (JC) of the eggs prior to deposit on the substrate and the community is then stable over much of the course of embryogenesis (Kerwin and Nyholm 2017). Even though the eggs do not receive parental protection, they still face potential threats from fouling microorganisms and/or predation. Many hypotheses have been proposed for the functional role of the ANG bacteria, including assisting in the sexual maturation of the host (Lum-Kong and Hastings 1992), contributing to the formation of the egg capsule (Buchner 1965, Drew 1911), or providing protection for the developing embryo against pathogenic or fouling organisms (Biggs and Epel 1991). However, while the antibacterial

activity of a few specific ANG/JC bacterial isolates, and of the whole ANG itself, have been demonstrated (Gromek et al. 2016, Gomathi 2010), egg defense remains untested.

Demonstrating function in the ANG or any number of host-microbe associations can prove difficult, especially since in many cases the microbial partners are only known from sequence-based community profiling. While determining the composition of microbiomes has become a relatively straightforward endeavor, understanding the interactions of the various members of these communities, and the overall function of a symbiosis, often lags behind and may be based on purely correlative studies. Tractable experimental models and diverse approaches are therefore needed to explore function in microbiome research.

The Hawaiian bobtail squid, *Euprymna scolopes*, offers a tractable experimental model for studying animal-bacteria symbioses (McFall-Ngai 2014; Kerwin and Nyholm, 2017). These squid are easily maintained in the laboratory for the duration of their life (McFall-Ngai 2014) with no effect on the composition of the ANG bacterial community (Kerwin and Nyholm 2017). The ANG is dominated by *Alphaproteobacteria* from the *Roseobacteraceae* family, mostly *Leisingera* sp. (Collins et al. 2012, Kerwin and Nyholm 2017). This community is also unique among ANG cephalopods in containing a large contingent of *Verrucomicrobia* (Collins et al. 2012, Kerwin and Nyholm 2017). The ease of maintaining bobtails in the lab and the stability of their ANG community both in the lab and throughout embryogenesis makes this system ideal for investigating the function of the ANG bacteria in cephalopods.

Here we present evidence of the function of a complex bacterial consortium, and integrate knowledge of community composition, functional ecology, chemistry, and natural product discovery to begin to understand the roles of bacterial members within the symbiosis. Using antibiotic treatment and egg manipulation, we demonstrate that ANG bacteria from *E.*

scolopes likely assist in resistance to fouling fungi. Using natural product chemistry, we examine the differential anti-fungal activity of organic extracts from numerous ANG and JC isolates against potential fungal pathogens, from within and outside the host's natural habitat. Finally, we propose a model for egg defense in ANG cephalopods.

Materials and Methods

Antibiotic clutch experiments

Squid were collected from a protected sandflat in Maunalua Bay, Oahu, HI (21°26'3.36"N, 157°47'20.78"W) and were shipped to Connecticut to be maintained in aquaria. Egg clutches from these squid were split into similarly sized sections, and placed in aerated filter-sterilized seawater (FSSW). Clutch segments were treated with 25 µg/ml chloramphenicol or an antibiotic cocktail (25 µg/ml each of penicillin G, kanamycin, spectinomycin, streptomycin, and gentamicin), and were monitored over a four-week period with daily water changes and fresh antibiotics. The viability of each clutch section was determined once hatching was complete (two days without additional juveniles produced) by dividing the number of unhatched eggs by the number of total eggs (number of unhatched eggs added to the number of juveniles produced). Health of a subset of hatched juveniles was checked by inoculating the water with the light organ symbiont, *V. fischeri*, and determining whether colonization occurred. Percent hatch was compared between untreated and antibiotic-treated clutches using a paired two-tailed t-test.

Egg component experiments

A bud cell collection method developed for *Fusarium oxysporum* (Diener 2005) was modified for use on *Fusarium keratoplasticum*. A culture of *F. keratoplasticum* was grown for 3-5 days in seawater-tryptone broth (SWT, Lee and Ruby 1992), shaking, at 30°C. The culture was

then strained through sterile gauze and centrifuged for 10 min at 3,200 Xg. The spores were then rinsed twice with sterile water and re-pelleted after each wash, with a final resuspension in one-fifth of the starting volume of sterile water. Bud cells were quantified using a haemocytometer and correlated with OD_{530} to determine a conversion factor of $3x10^6$ bud cells/ml/OD_{530}. Refrigeration for up to one week was found to have no effect on the number of bud cells present, or on the viability of the bud cells (data not shown).

E. scolopes eggs from the same clutch were dissected using sterile forceps into their various components: intact eggs, eggs lacking outer capsule, and eggs lacking outer capsule and jelly coat. Dissections were completed at day 5 of embryogenesis, as earlier dissection prevented embryo development. These components were challenged with 10^4 *F. keratoplasticum* bud cells/ml over the course of embryogenesis, an order of magnitude higher than the eggs are likely to encounter naturally (Goeke 2010). Eggs were maintained in FSSW with water changes and fresh spores added every 2-3 days, and were kept on a rocker to provide aeration.

Transmission electron microscopy

A freshly deposited *E. scolopes* clutch was divided into sections. One section was left untreated, one was treated with 20 µg/ml of the antibiotic chloramphenicol, and one was treated with 0.13 µl/ml of 95% ethanol, the chloramphenicol vehicle. Sections were maintained in aerated FSSW, which was changed daily. By day 21 a thick fungal biofilm had formed on the chloramphenicol-treated eggs. Decapsulated eggs were prepared for TEM following established protocols (Kerwin and Nyholm 2017, Collins et al. 2012).

Antibiotic-treatment effects on bacterial communities

Antibiotic-treated clutch segments were maintained in a laminar flow hood to prevent fungal infection and were otherwise treated as those described above. Throughout the course of

experiments five eggs from the same clutch (n=3) were sampled on Days 0, 10 and 21, the JC was dissected out, and JCs were surface sterilized by washing quickly in ethanol, and homogenized in filter-sterilized squid Ringer's solution (FSSR, Collins et al. 2012), then plated on SWT to quantify JC bacterial abundance. Differences in bacterial load between antibiotic-treated JCs and untreated JCs were analyzed via two-tailed t-tests for each time point.

Sections of the biofilm were homogenized in FSSR. Biofilm bacterial DNA was extracted using the DNeasy Blood and Tissue kit according to the manufacturer's protocol (Qiagen, Valencia, CA). DNA concentration was determined using the Qubit® dsDNA High Sensitivity assay (ThermoFisher Scientific Inc., Waltham, MA) and averaged 1.13 ng/µl ± 0.79 ng/µl. The V4 region of the 16S rRNA gene was sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) following established protocols (Kerwin and Nyholm 2017, Benjamino et al., 2016, Nelson et al., 2014). Sequencing data was analyzed using QIIME (Caporaso et al., 2010) and NMDS plots of Bray-Curtis beta-diversity analyses were created in R using the VEGAN package (Oksanen et al. 2016) as previously described (Kerwin and Nyholm 2017). Sequencing data was compared to ANG/JC community data previously published (Kerwin and Nyholm 2017) under the accession number ENA PRJEB14655.

Fungal isolation and characterization

Fungi from antibiotic-treated clutch biofilms were isolated on inhibitory mold agar with gentamicin and underwent morphological characterization and multi-locus sequence typing using four genes: TEF1-α, RPB2, ITS rDNA, and the 5' end of LSU rDNA (O'Donnell 2010).

A clutch deposited by a wild female while maintained in a tank with natural Hawaiian seawater and Hawaiian sand was divided into two sections. One section was treated with 25 µg/ml of an antibiotic cocktail (chloramphenicol, penicillin G, kanamycin, spectinomycin,

streptomycin, and gentamicin) for one week while in an aerated beaker with daily water changes. The other section was left untreated. After one week of treatments both sections were transferred back to their original tank with running seawater for the remainder of embryogenesis. The untreated section had 94% viability, while the antibiotic-treated section had 10% clutch viability and developed a biofilm. The outer capsule of an egg that did not hatch and a decapsulated egg containing a viable embryo from that section were both plated onto SWT. Fungal colonies were then isolated on inhibitory mold agar with gentamicin and underwent morphological characterization and multi-locus sequence typing using four genes: TEF1-α, RPB2, ITS rDNA, and the 5' end of LSU rDNA (O'Donnell 2010).

Fungal diversity was examined for Hawaiian sediment and seawater samples previously extracted and characterized for their bacterial communities (Kerwin and Nyholm 2017), and of egg capsules extracted using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) as previously described for ANGs (Kerwin and Nyholm 2017). The ITS2 region was amplified and sequenced with ITS3/4 (White 1990) using the same process as described above for the V4 region of the 16S rRNA gene. Sequencing data was analyzed using de novo methods alone in QIIME (Caporaso et al., 2010).

Results and Discussion

Egg clutches treated with antibiotics, which effectively reduced the bacterial load of the JC by 98% across embryogenesis (Figure 2I), developed a thick biofilm that typically appeared between days 8-10 of embryogenesis, and which did not affect untreated clutches kept under the same conditions (Figure 2). Biofilm growth on the eggs resulted in very low hatch rates, averaging 9% ± 13%, compared to 58% ± 33% hatch for untreated clutches (Figure 3). Bud cells and hyphae were found penetrating the JC layers of antibiotic-treated eggs, which also appeared

to contain fewer bacterial cells compared to JC layers of untreated eggs (Figure 2C, 2F). When the biofilm was examined further it was found to be dominated by fungal hyphae interspersed with bacterial cells (Figure 2G-H). Fungal isolates from the biofilm were analyzed via MLST and determined to be *Fusarium keratoplasticum*, a recently described species arising from the *Fusarium solani* species complex (FSSC), specifically haplotype FSSC-2g (Figure 4, Short et al. 2013). *F. keratoplasticum* is dominant in plumbing biofilms, explaining the occurrence of this fungus as a common lab contaminant (Short et al. 2014). Eggs treated with antibiotics and maintained in Hawaiian seawater also developed fungal biofilms dominated by *F. keratoplasticum*, demonstrating that this type of fungus is also present in Hawaii. Four Hawaiian seawater isolates of *F. keratoplasticum* were all identified as haplotype FSSC-2xx, a closely related strain to the lab-isolated FSSC-2g (Figure 4).

Fusarium solani is a filamentous ascomycete fungus (Richards 2012). The *Fusarium solani* species complex (FSSC) contains numerous cryptic species including at least 45 phylogenetically distinct species (O'Donnell 2008, 2010). The species designation of *F. keratoplasticum* has recently been assigned to the FSSC-2 clade (Short et al. 2013). These ubiquitous and opportunistic terrestrial and marine pathogens cause fusariomycosis by infecting tissue that is either dead or damaged (Richards 2012), and can cause life-threatening infections (O'Donnell 2008). The FSSC has been found to infect people (Yera et al. 2003), livestock, agricultural products, marine animals, and marine eggs (O'Donnell 2008, 2010). Within the FSSC, *F. keratoplasticum* is one of the most common causative agents of fungal keratitis and a significant opportunist for human infections (Tupaki-Sreepurna et al. 2016). Current antifungals are largely ineffective against the FSSC (O'Donnell 2008), including *F. keratoplasticum* (Taj-

Aldeen et al. 2016, Tupaki-Sreepurna et al. 2016), and surgical intervention is commonly required (Yera 2003).

The bacterial community of the biofilm which formed on the eggs during antibiotic treatment was also extracted and profiled and was found to be dominated by *Gammaproteobacteria* and *Alphaproteobacteria*, and to also contain nine other bacterial classes (Figure 5). When some host animals are stressed, the symbiotic relationship can break down and the symbionts are expelled (Ralph et al. 2001). However, the biofilm community was distinct in composition from that of the ANG/JC (Figure 5, Kerwin and Nyholm 2017), indicating that the bacteria present were not solely being expelled from the eggs. The biofilm community composition also varied widely between biofilm samples, demonstrating that this community was not stable, but more random (Figure 5C). This biofilm community could consist of environmental bacteria for which the biofilm provided a suitable substrate, or could be opportunistic pathogens of the eggs. Nine bacterial isolates from various biofilms were obtained, all *Gammaproteobacteria*, and will be used for future testing.

Eggs were also maintained under conditions that did not promote fungal growth to investigate whether the antibiotic treatment itself was negatively impacting the developing embryos. Eggs were placed in a laminar flow hood or were maintained at a low temperature, in both cases in filter-sterilized seawater. *F. keratoplasticum* FSSC-2g is unable to grow at temperatures under 15°C (Kerwin, pers. obs.), so when eggs were maintained between 15°-20°C any fungal bud cells present should not have been able to germinate. When eggs were treated with an antibiotic cocktail (penicillin G, kanamycin, spectinomycin, streptomycin, and gentamicin) under either of these conditions the embryos hatched at similar rates as those from untreated eggs and were successfully colonized with the light organ symbiont, *Vibrio fischeri*

(Figure 3), demonstrating that this treatment did not affect embryonic development. However, when eggs were treated with chloramphenicol and maintained in a laminar flow hood, embryonic development ceased prior to embryos being fully formed and no hatching was observed (Figure 6). This negative result indicates that the chloramphenicol itself interfered with embryo development in this species, although other studies have shown that juvenile squid and adults are not negatively affected by chloramphenicol at similar doses (Lamarcq and McFall-Ngai 1998, Nyholm et al. 2002, Nyholm et al. 2009, Schleicher et al. 2014).

Biofilms visible to the naked eye typically appeared on antibiotic-treated clutches between days 8-14 of embryogenesis (n=17 clutches, Figure 3). However, fungal hyphae appeared on the egg surface as early as day 3 of embryogenesis (Figure 7B) and grew into a biofilm by day 10 (Figure 7C-E), which then continued to grow until completely enveloping the clutch by day 15 (Figure 7F-G). In contrast, no fungal hyphae appeared on untreated eggs from the same clutch at any point in development (Figure 7A, H-M).

Eggs were dissected into various components and challenged with a 10^4 *F. keratoplasticum* bud cells/ml suspension to begin to discern the effects of the bacterial community from potential host factors of the developing embryo (Figure 8). By day 18 of embryogenesis, when the embryos were mature and on the verge of hatching, challenged eggs that were left intact showed few signs of fungal infection (Figure 8B). A few hyphae were noted on the surface of these eggs, but the level of hyphae appeared more reminiscent of the day 3-6 biofilm from the antibiotic-treated eggs (Figure 7B-C), and was not established enough to impact the development of the embryos. When the outer capsule was removed, leaving the JC and its bacterial community intact, similar results were observed to the intact eggs (Figure 8D). However, when we removed both the outer capsule and the JC, leaving only the developing

embryo within its yolk sac, the eggs developed a thick fungal biofilm that took over the eggs and prevented the embryos from developing (Figure 8F). This experiment, in conjunction with the antibiotic experiments discussed above, provides evidence that the JC and its bacterial community are essential to the prevention of fungal fouling in *E. scolopes* eggs.

Bacterial protection from fungi has been documented in several invertebrate systems. In the Oriental shrimp, *Palaemon macrodactylus*, and in the American lobster, *Homarus americanus*, embryos are coated in bacteria which produce compounds that inhibit the growth of the fungus *Lagenidium callinectes* (Gil-Turnes et al. 1989; Gil-Turnes and Fenical 1992). When hydra, *Hydra vulgaris*, are treated with antibiotics to remove the resident epithelial microbiota, the animals develop a detrimental infection of the fungus *Fusarium* sp., and are rescued by complimenting with members of their original bacterial community (Fraune et al. 2015). Female beewolves, tribe Philanthini within the *Crabronidae*, apply bacterial symbionts from antennal reservoirs to the brood chambers of their larvae, which are then incorporated into the larval cocoon to protect against environmental mold fungi (Flórez et al. 2015). These examples demonstrate that diverse marine and terrestrial invertebrates can utilize bacterial products to ward off fungi.

In an attempt to examine the fungal diversity present in the natural environment of the bobtails, we used ITS2 primers to amplify fungal DNA from the seawater and sediment of Maunalua Bay, Oahu, HI, as well as from the egg capsule, which was dissected apart from the JC and yolk sac of eggs. We were unable to amplify any DNA product from any of the egg capsules attempted (n=16), indicating that if any fungal cells are present on the exterior of the eggs, they are below the limit of detection for PCR. Fungal DNA was amplified for both seawater (n=4) and sediment (n=9) samples. However, for both seawater and sediment samples the vast majority

of OTUs obtained resulted in no blast hit (on average 95.5% and 99.8% respectively), so the fungal diversity from the host's habitat remains to be determined. Marine fungi are in general poorly understood, leading to difficulties in matching sequencing data to known isolates.

Ninety-six bacterial isolates were obtained from both ANG and JC tissues which spanned several phylogenetic groups (52 *Alphaproteobacteria*, 21 *Gammaproteobacteria*, and 3 *Flavobacteriia*; the *Verrucomicrobia* are not yet culturable), and those isolates were used to obtain 103 organic extracts in collaboration with Dr. Marcy Balunas and Samantha Gromek. Thirty-six of these extracts were tested for antifungal activity against three strains of *F. keratoplasticum* (FSSC-2g from original antibiotic testing, FSSC-2xx from egg biofilm in Hawaiian seawater, and FSSC-2d, a lab isolate obtained from the Fungal Testing Laboratory in San Antonio, TX) and against the human fungal pathogen *Candida albicans*. These tests demonstrated that a number of extracts inhibited either *F. keratoplasticum* or *C. albicans* (Figure 9). Interestingly, the pattern of inhibition differed depending on the fungal strain tested. While the three strains of *F. keratoplasticum* were similarly inhibited by the extracts, a different set of extracts inhibited *C. albicans*. Overall 15 extracts were able to inhibit at least one of the *F. keratoplasticum* strains (<50% percent control activity), while nine were able to inhibit *C. albicans*, but only six extracts were capable of inhibiting both types of fungi (Figure 9).

The differential activity of extracted compounds from ANG/JC bacterial isolates against *F. keratoplasticum* and *C. albicans*, in combination with previously reported ANG/JC isolate activity against certain other bacterial strains (Gromek et al. 2016), has led us to develop a model for the function of this complex symbiosis (Figure 10). The ANG bacterial community is composed of functionally diverse members, even when those members are closely related phylogenetically (Kerwin and Nyholm 2017, Gromek et al. 2016, Collins et al. 2015, this study).

The functional diversity allows the community to protect the developing embryo from a variety of potential threats throughout the duration of embryogenesis. During the three-week embryogenesis period *E. scolopes* eggs receive no parental care, and exist in an environment where fouling is common on all available substrates. Within the JC bacterial community, a subset of strains will produce secondary metabolites that inhibit one fungal strain. Another subset of strains will be active against a different fungus as is shown here. Still other subsets will be active against various potential bacterial colonizers (Gromek et al. 2016). Potentially other subsets of the JC symbiotic community could prevent colonization by various marine larvae and algae. The JC material itself can inhibit ciliary beat and could prevent certain protists from invading the egg (Atkinson 1973). Thus, this complex JC environment offers a multifaceted defense of the embryo throughout development as a substitute for parental care.

The ability of marine eggs to survive to adulthood in an environment filled with potential biofoulers depends on the presence of one or more defensive mechanisms. Some species depend on parental protection, others compensate for low survival by laying a large number of eggs, and still others utilize chemical protection, either by the host itself or from various symbionts. The questions of why squid maintain such an ANG and why females deposit that bacteria into their eggs have been asked since the ANG was first described in *Doryteuthis pealeii* in 1909 (Drew 1911; Pierantoni 1918, Williams 1909). Many hypotheses have been proposed, including that the ANG contributes to formation of the eggshell (Buchner 1965, Drew 1911), and that the ANG material influences sexual maturation of the squid (Lum-Kong and Hastings 1992). JC bacteria were first hypothesized to serve a protective function in squid eggs in *Doryteuthis opalescens* (Biggs and Epel 1991), and this hypothesis has since been proposed for various species of cephalopods (Barbieri 1997, Grigioni 2000, Gomathi 2010, Pichon 2005, Collins et al. 2012). In

this study, we demonstrate for the first time that the JC bacterial community in *E. scolopes* prevents fungal fouling and resulting embryo death.

Our research is the first study to experimentally link this defensive function to the ANG/JC symbiotic bacterial community. The similarity of this symbiosis and conservation of ANG bacterial members to other cephalopods indicates that the function of this organ may be conserved among other cephalopods. The phylogenetic and functional diversity of this symbiotic community offers a wide range of targets for future avenues of research, including the investigation of the community for anti-algal activity, anti-predation activity, and anti-bacterial activity. In addition, future research can examine whether certain extracts work synergistically against fungal and other threats within the system. The *E. scolopes* ANG bacterial consortium provides a tractable system to explore functional specificity in a marine symbiosis.

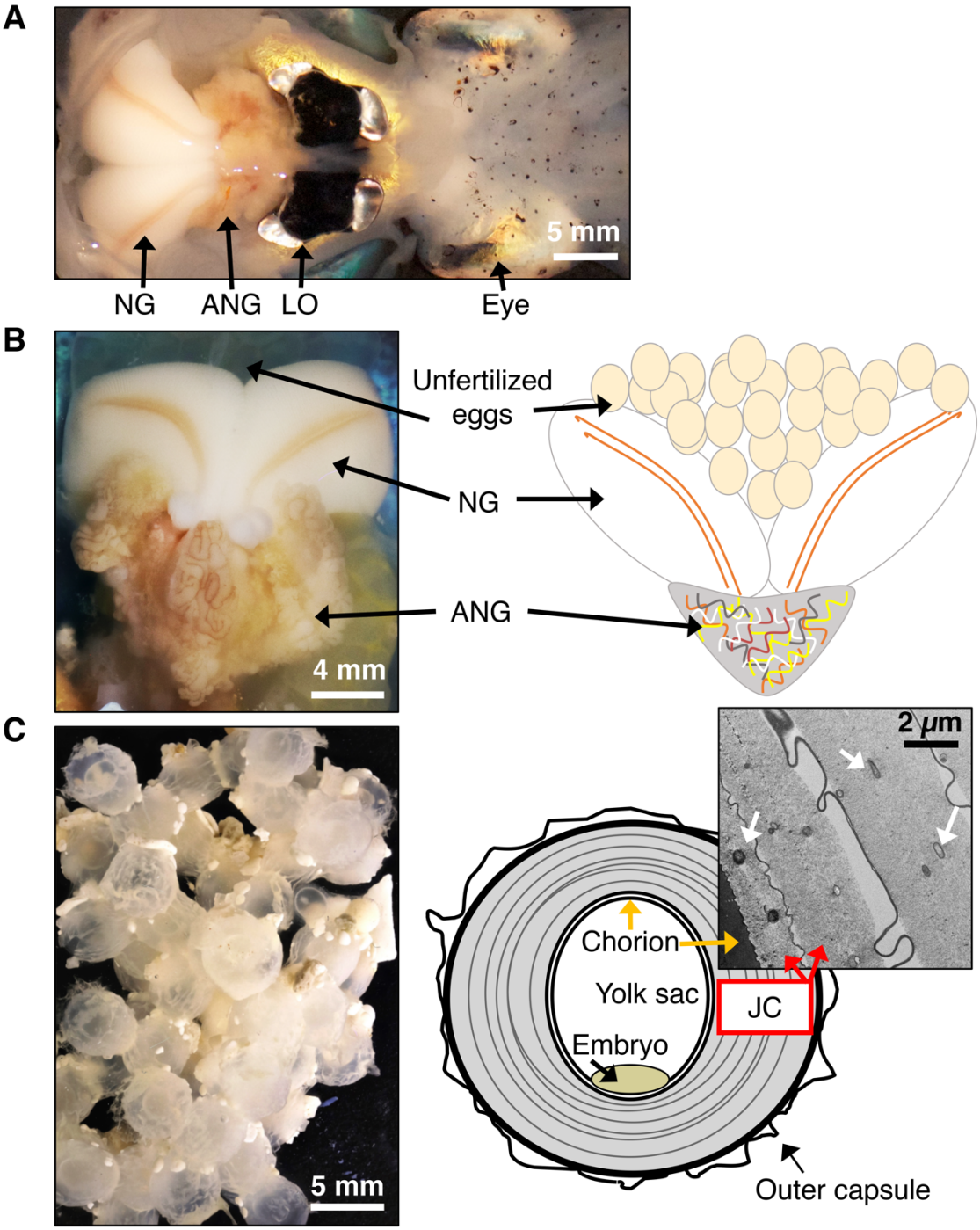


Figure 1. Ventral dissection of a female *E. scolopes* showing location of reproductive system organs relative to the light organ and eye of the squid (A). Detailed image and diagram of female reproductive tract (B) and egg (C). Inset is electron micrograph of Day 0 JC, bacteria indicated by white arrows. NG = nidamental gland; ANG = accessory nidamental gland; JC = jelly coat; LO = light organ.

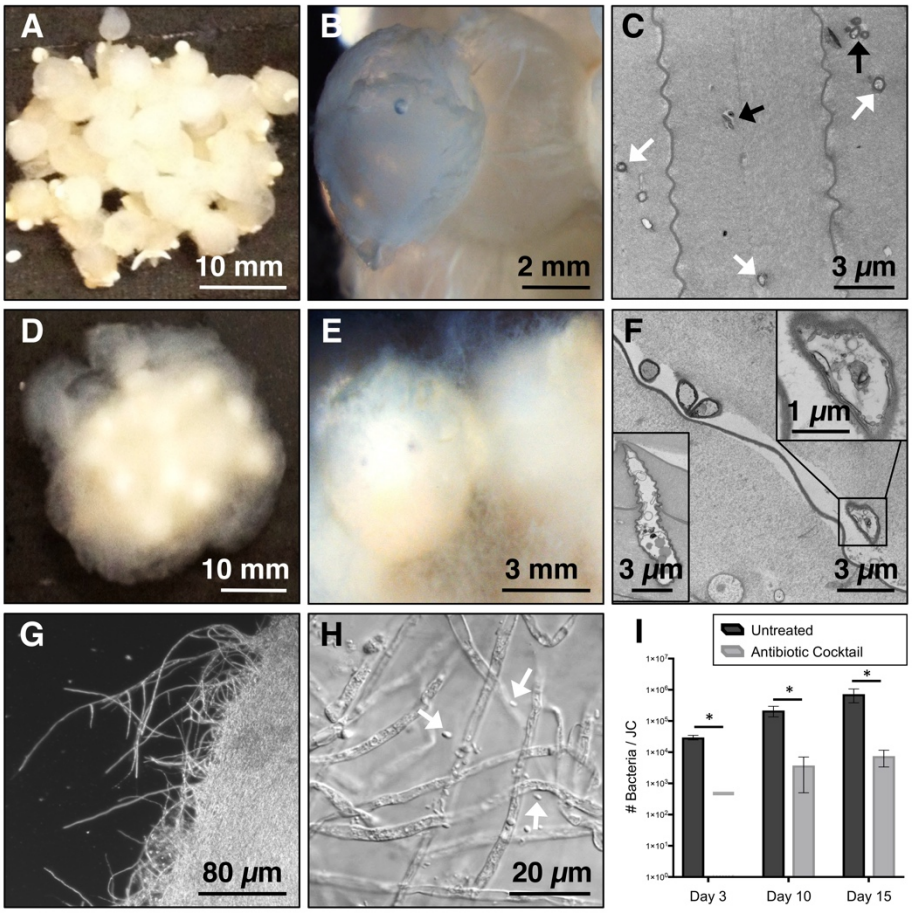
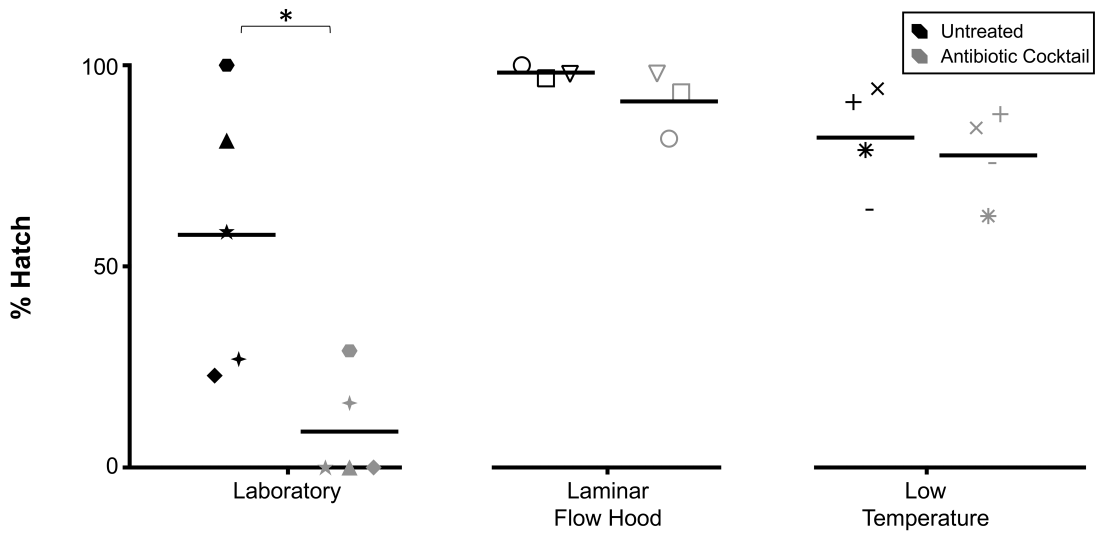


Figure 2. Treatment of clutches with antibiotics led to the formation of a fungal/bacterial biofilm. Clutches were either left untreated (A-C) or were treated with antibiotics (D-F). The biofilm was composed of fungal hyphae interspersed with bacterial cells (G-H, white arrows = bacteria). Electron micrographs of JCs from eggs at Day 21 of embryogenesis show untreated egg JCs contain both single (white arrows) and small clusters of bacterial cells (black arrows, C). The antibiotic-treated egg JCs contain numerous fungal spores and hyphae (insets, F) as well as degraded bacterial cells (not shown). Representative images of antibiotic trials as described in Figure 3. Treatment of eggs with an antibiotic cocktail resulted in a 98% reduction in the amount of culturable strains present in the JC (n=3 clutches, I). After three days of treatment no bacteria were detectable in the antibiotic-treated JCs (line represents limit of detection, 500 bacteria/JC), a 98% reduction in bacterial load (t_4=5.854, p=0.004). The bacterial load of both the untreated and the antibiotic-treated clutch segments increased over embryogenesis, but a significant reduction was still found both at day 10 (t_4=3.001, p=0.04) and at day 15 (t_4=2.828, p=0.047) of embryogenesis. Antibiotic cocktail included penicillin G, kanamycin, spectinomycin, streptomycin, and gentamicin, each at a concentration of 25 µg/ml.



Experiment Environment	Treatment	# of clutches	Average Eggs/Clutch	Day of Biofilm Appearance	Days Hatchlings Appeared	Average % Hatch
Laboratory	Antibiotic Cocktail	5	37	8-14	17-24	9% ± 13%
	Untreated	5	27	-	17-30	58% ± 33%
Laminar flow hood	Antibiotic Cocktail	3	57	-	14-25	91% ± 8%
	Untreated	3	48	-	13-23	98% ± 2%
Low temperature (15°-20°C)	Antibiotic Cocktail	4	43	-	23-31	78% ± 11%
	Untreated	4	33	-	23-31	82% ± 14%

Figure 3. Treatment of eggs with antibiotics and subsequent development of a fungal biofilm significantly reduced hatch of juveniles. Eggs were treated with 25 µg/ml of antibiotic cocktail (t_4=3.572, p=0.023). Hatching levels were unaffected when eggs were treated with antibiotic cocktail and maintained in a laminar flow hood (t_2=1.289, p=0.326) or under low temperatures (15°-20°C, t_3=0.738, p=0.514) to prevent fungal growth. Antibiotic cocktail included penicillin G, kanamycin, spectinomycin, streptomycin, and gentamicin, each at a concentration of 25 µg/ml. Data point shape reflects eggs taken from the same initial clutch.

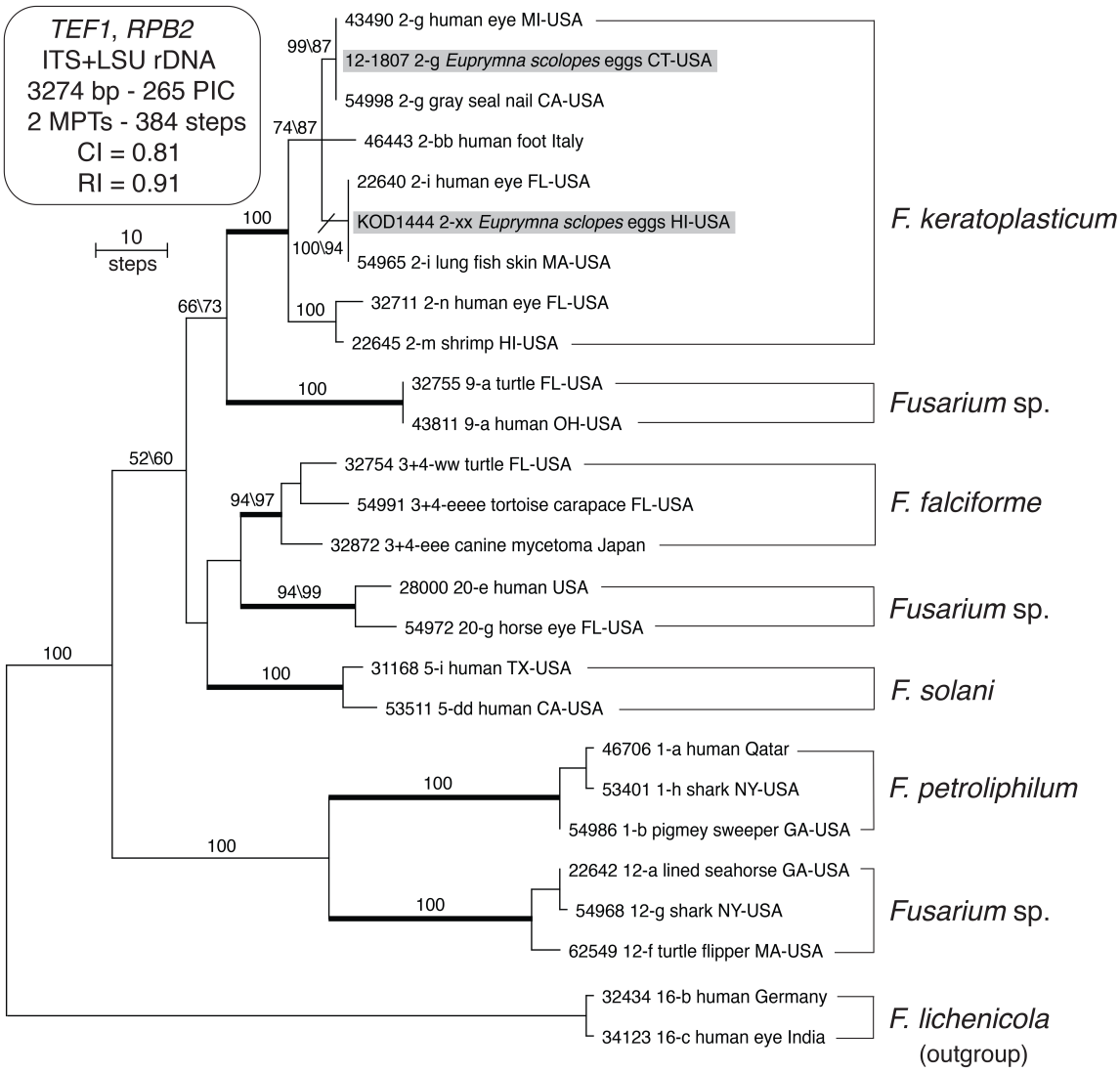


Figure 4. Maximum parsimony (MP) analysis of multilocus DNA sequence data from 26 isolates within the *Fusarium solani* species complex (FSSC), including two isolates of clutch biofilm, highlighted in grey, from antibiotic-treatment experiments in Connecticut and Hawaii. Sequences of two soybean pathogens were used to root the phylogram. Arabic numbers and lowercase roman letters identify the species and sequence types, respectively, within the ingroup. Numbers above internodes indicate MP bootstrap support. PIC, parsimony informative character; CI, consistency index; RI, retention index.

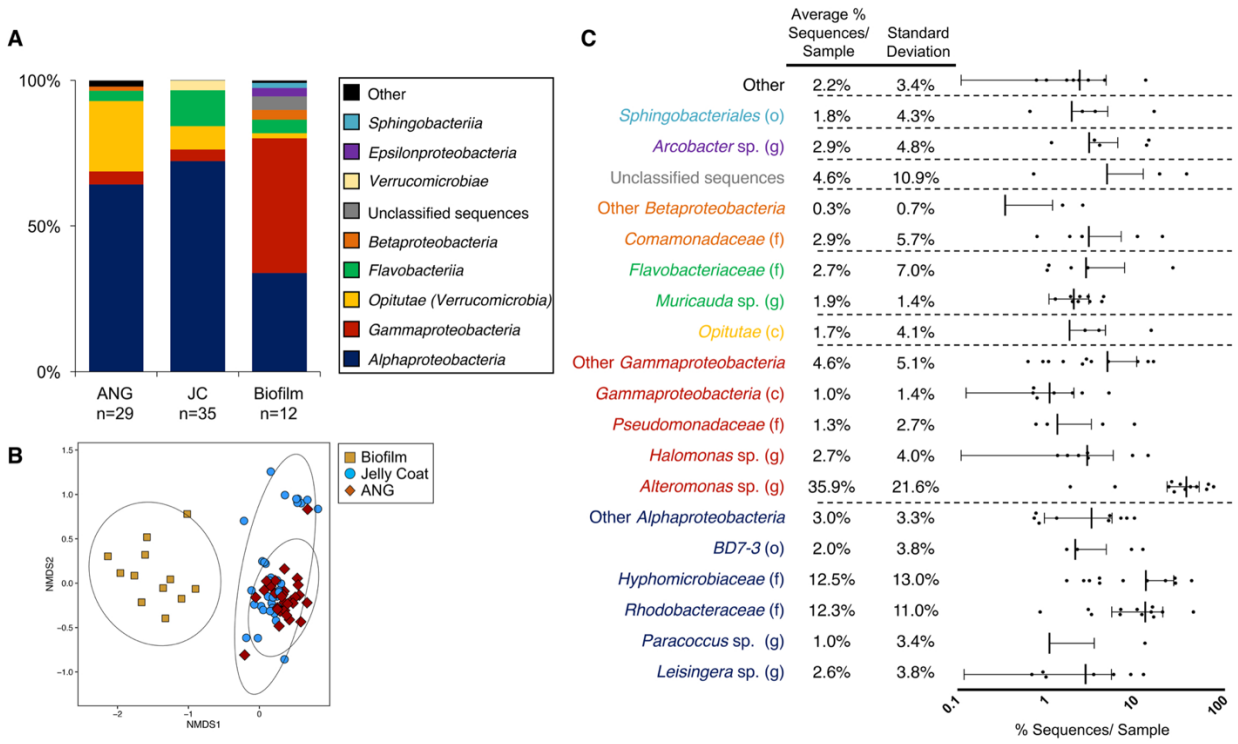
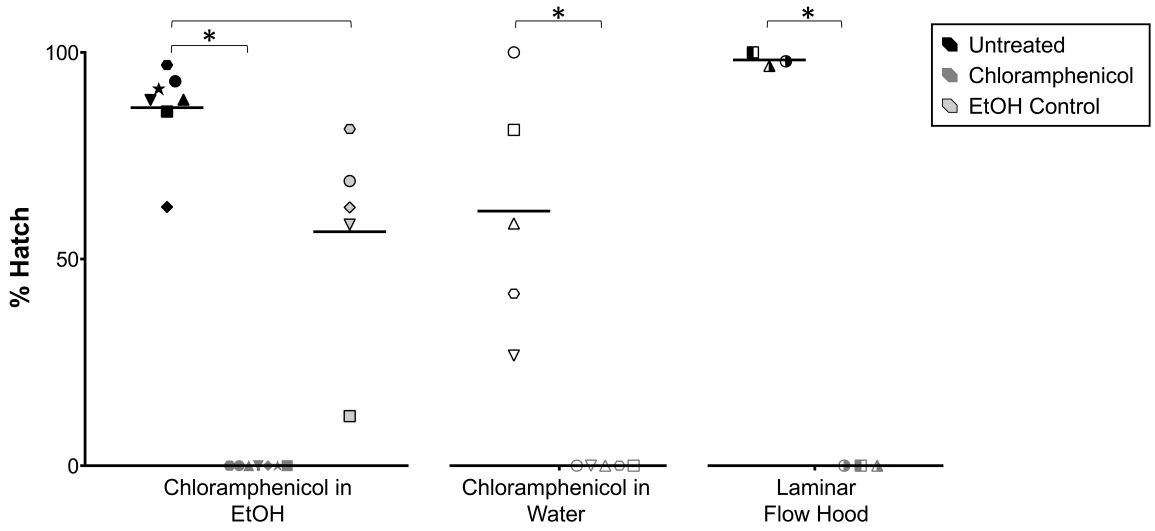


Figure 5. The biofilm bacterial community (n=12) is dominated by *Gammaproteobacteria* (A) and is different from that found in the ANG and JC (B). The relative abundances of taxa that make up the biofilm community vary substantially between samples (C). Taxa presented at the finest level obtained, c – class; o – order; f – family; g – genus. Mean % sequences/sample represented by thick bar, standard deviation represented by thin bars. Scatter plot is presented on a log scale to demonstrate variation for taxa present at lower average abundances. Other includes taxa present at <1% of the average biofilm: unclassified *Bacteria*, *Stramenopiles* (o), *Pirellulaceae* (f), unclassified *Proteobacteria* (p), *Myxococcales* (o), and *Nannocystaceae* (f). NMDS plot based on Bray Curtis metric of Beta diversity demonstrates that the community composition of the biofilm is distinct from that of the ANG/JC (C). ANG and JC data were previously published (Kerwin and Nyholm 2017).



Experiment Environment	Treatment	# of clutches	Average Eggs/Clutch	Day of Biofilm Appearance	Days Hatchlings Appeared	Average % Hatch
Laboratory	Chloramphenicol (20 μg/ml)	7	26	11-19	-	0%
	Ethanol Control	5	29	-	23-27	57% ± 26%
	Untreated	7	38	-	20-27	87% ± 11%
	Chloramphenicol (25 μg/ml)	5	37	10-12	-	0%
	Untreated	5	27	-	17-28	62%
Laminar flow hood	Chloramphenicol (25 μg/ml)	3	45	-	-	0%
	Untreated	3	48	-	13 - 23	98%

Figure 6. Treatment of eggs with chloramphenicol led to the development of a fungal biofilm, but also prevented eggs from developing. Eggs were treated with 20 μg/ml of chloramphenicol dissolved in ethanol (t_6=20.47, p<0.0001), or with 25 μg/ml of chloramphenicol dissolved in seawater (t_4=4.668, p=0.01), and developed a biofilm in both cases. Treatment with ethanol alone did not significantly reduce juvenile hatching (t_4=2.33, p=0.08). However, hatching levels were affected when eggs were treated with chloramphenicol and maintained in a laminar flow hood (t_2=103.6, p<0.0001) to prevent fungal growth. Data point shape reflects eggs taken from the same initial clutch.

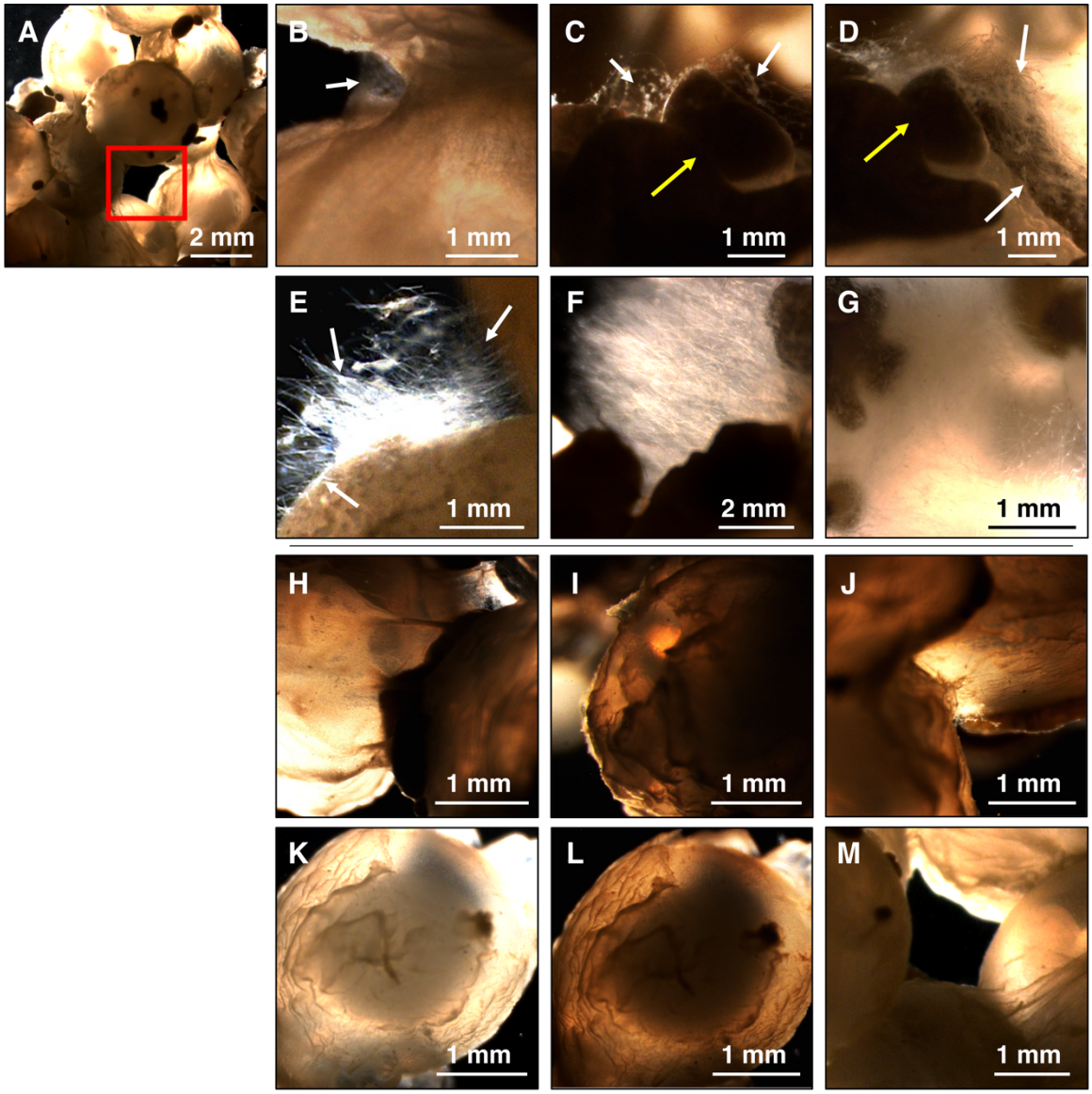


Figure 7. A time series illustrating biofilm development over embryogenesis. Clutches were either treated with antibiotics (B-G) or were left untreated (H-M). Time series images focus on the surface of 1-2 eggs, such as the segment indicated by the red box (A). On day 3 a few potential hyphae could be seen (B, white arrow), those hyphae increased in number on days 6 (C) and 8 (D), until forming a small biofilm on day 10 (E), which then increasingly took over the clutch on days 13 (F) and 15 (G), as opposed to untreated eggs which by day 15 (A, M) showed no signs of fungal hyphae. The same grain of sand can be seen in C/D, indicated by yellow arrows. Untreated eggs also shown throughout embryogenesis: day 3 (H), day 6 (I), day 8 (J), day 10 (K), day 13 (L), day 15 (M). Representative images of untreated and antibiotic-treated eggs taken from the same original clutch.

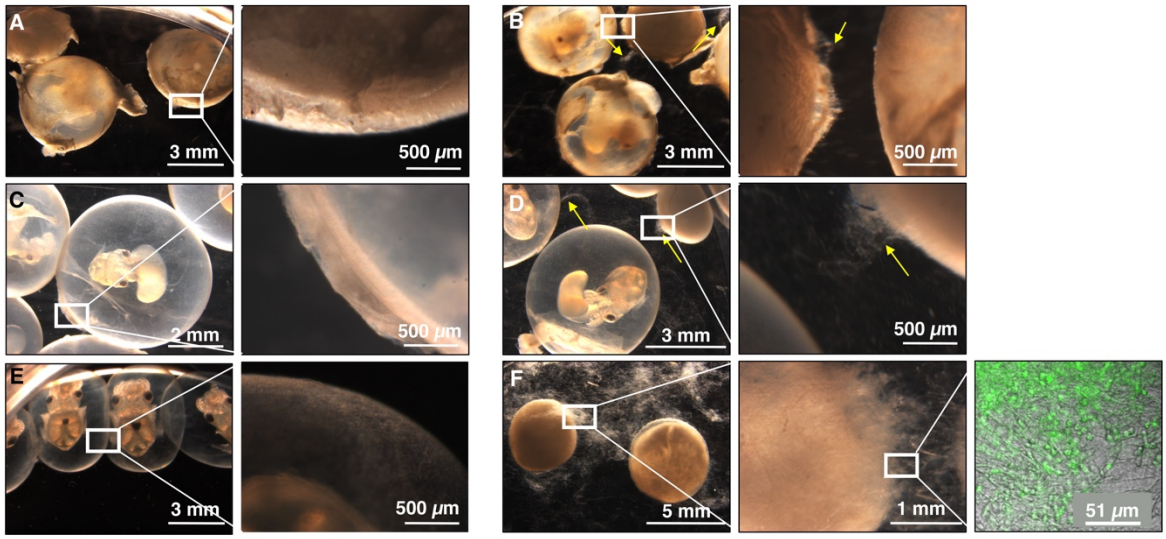


Figure 8. *E. scolopes* embryos lacking jelly coat protection are more susceptible to fungal infection (n=4 trials, 8-10 eggs/treatment). Eggs were left untreated (A/C/E) or were challenged with 10^4 *F. keratoplasticum* bud cells/ml for 18 days (B/D/E). Eggs were left intact (A/B), or were decapsulated, leaving only the jelly coat and developing embryo (C/D) or only the developing embryo (E/F). Inset in F stained with Syto9 nucleic acid stain and visualized on confocal microscope. Yellow arrows indicate fungal hyphae.

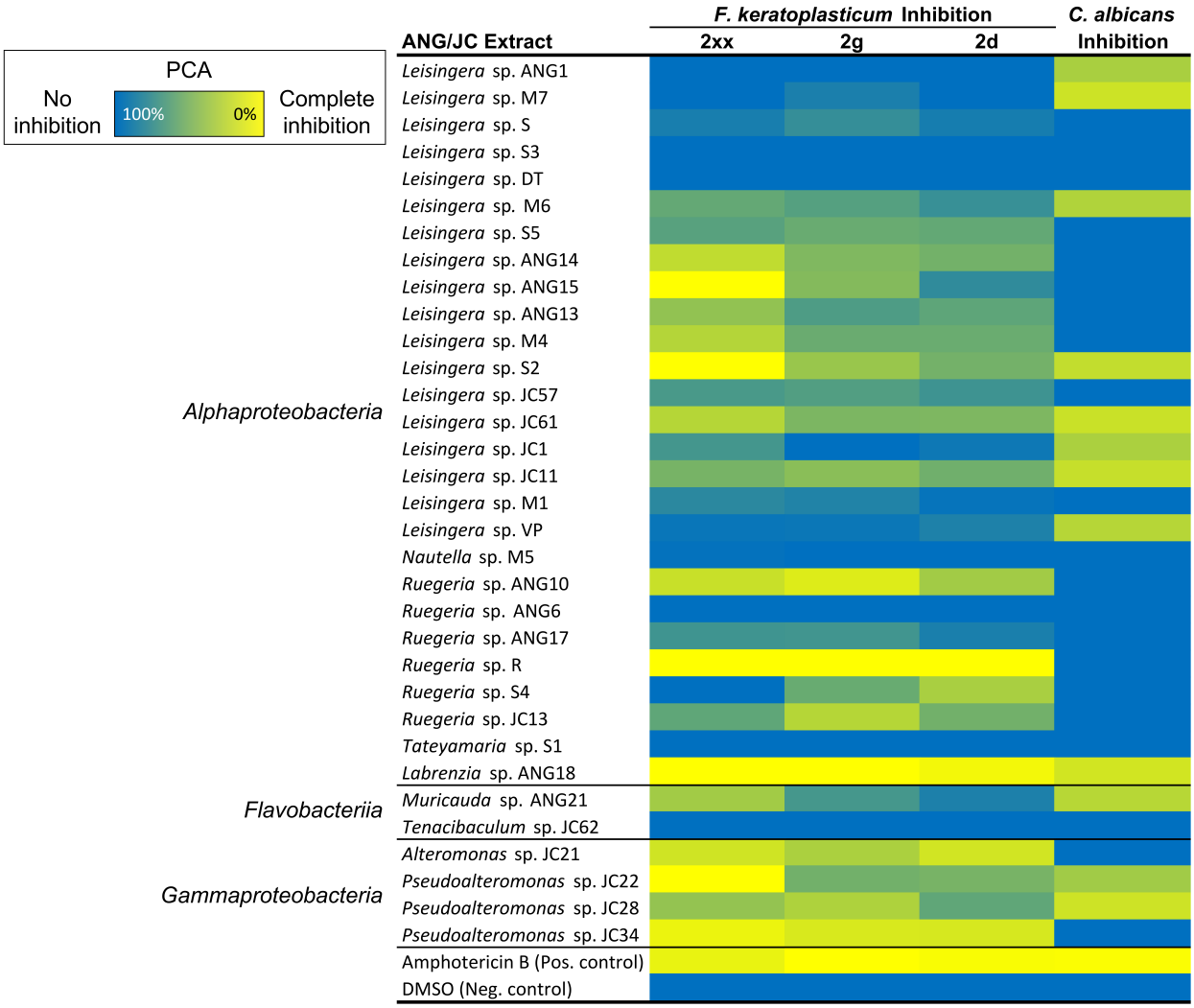


Figure 9. *E. scolopes* ANG and JC bacterial isolate extracts inhibit two types of fungi. Results from inhibition assays using organic extracts from ANG/JC isolates (500 µg/ml) against three strains of *F. keratoplasticum* (OD_{600} 0.15-0.17) and the human fungal pathogen *Candida albicans* (OD_{600} 0.08-0.1). Inhibition was measured spectrophotometrically after 14 hr incubation, presented as average percent control activity (PCA) compared to DMSO control.

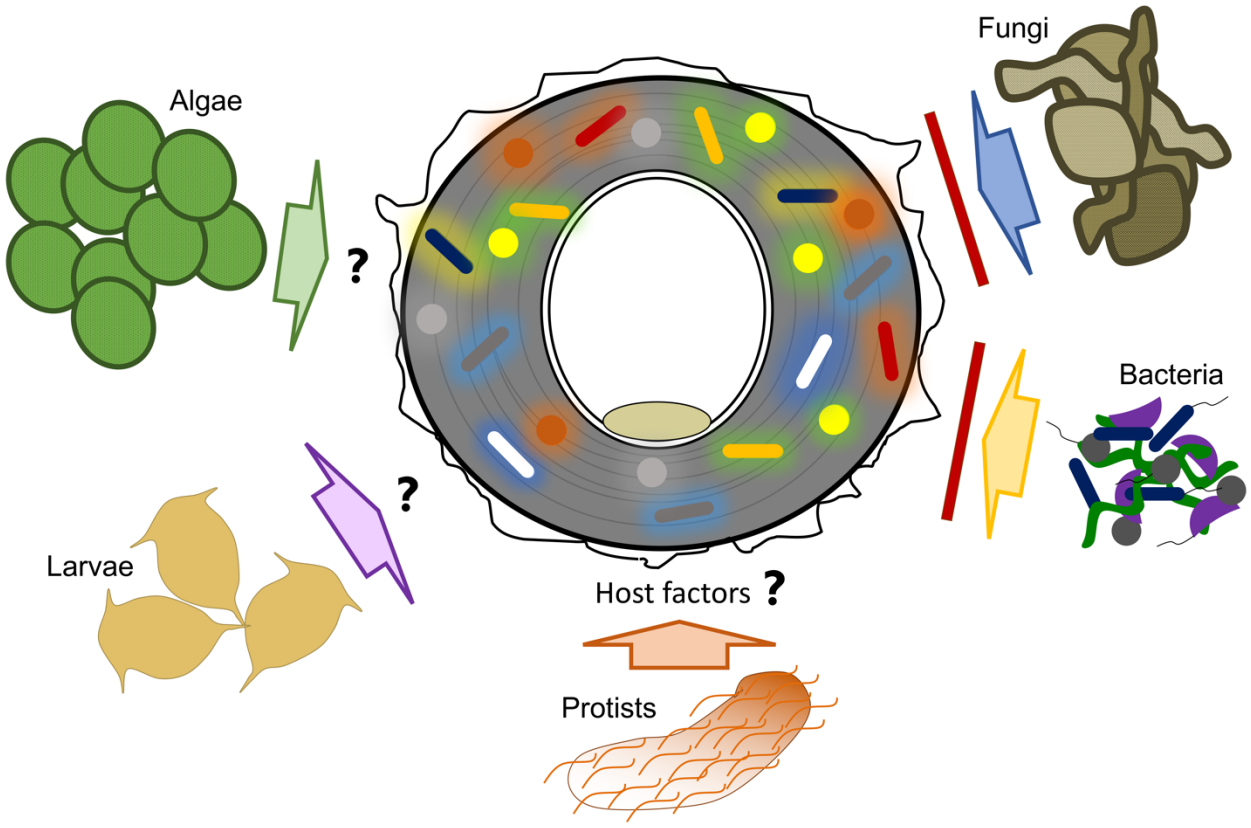


Figure 10. Model of embryonic defense in *E. scolopes*. Subsets of the JC bacterial community produce various secondary metabolites to prevent bacterial colonization (Gromek et al. 2016) or fungal fouling (this study). Potentially, other subsets of the community could ward off marine larvae or algae from fouling the egg surface. The JC material itself has been shown to inhibit ciliary beat and may protect the embryo from certain protists (Atkinson 1973). In this model, the JC is crucial to embryonic defense, with both host-derived and bacterial components playing a role.

Chapter 4

Development of the ANG and associated bacterial community over the course of female *Euprymna scolopes* maturation

Acknowledgments

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Abstract

The accessory nidamental gland (ANG) of the Hawaiian bobtail squid, *Euprymna scolopes*, is a female reproductive symbiotic organ which contains a complex bacterial community. Once this community is deposited in the jelly coat of the squid's eggs, it plays an important defensive role, preventing fungal fouling and bacterial infection. While the community is known to be conserved across a wild population, and to be unaffected by laboratory-maintenance, little is known about how the community develops. Here we demonstrate that the bacterial community changes as the squid approaches maturity, increasing in community evenness, and shifting from a *Verrucomicrobia*-dominated community to an *Alphaproteobacteria*-dominated community. We also explore the effect of laboratory raising on ANG bacterial community composition, providing further evidence for the environmental transmission of this symbiosis. Finally, we examine the formation of the ANG tissue itself, and demonstrate that this organ develops in a similar manner to that shown for other cephalopods, and potentially to that of the light organ in *E. scolopes*. Understanding the manner of development of this important symbiotic organ will extend the use of this symbiosis as a model for complex marine symbiosis, and could eventually allow us to manipulate the bacterial community composition while testing the multifaceted functions of the community.

Introduction

Embryogenesis of Hawaiian bobtail squid (*Euprymna scolopes*) eggs lasts for approximately three weeks (Lee et al. 2009, Arnold et al. 1972). Juvenile *E. scolopes* hatch with a fully formed light organ primed for colonization by *Vibrio fischeri*, which occurs within twelve hours of hatching (reviewed by McFall-Ngai 2014). However, these hatchling squid lack all reproductive organs. While growing to sexual maturity female squid must form their accessory

nidamental gland (ANG), a symbiotic secretory organ which contains a diverse consortium of bacteria. In adult females, the ANG is composed of a tangled network of tubules, each of which contains its own dominant bacterial taxon (Collins et al. 2012). The symbiotic community in adult females is dominated by *Alphaproteobacteria* and *Verrucomicrobia*, with a smaller proportion of *Gammaproteobacteria*, and *Flavobacteriia* (Kerwin and Nyholm 2017, Collins et al. 2012). This bacterial community is deposited into the jelly coat of *E. scolopes* eggs, where it serves a protective function for the developing embryos (Gromek et al. 2016, Kerwin and Nyholm 2017, Chapter 3).

Microbial symbionts can be passed to the next host generation by two mechanisms. Either the microbes are transferred vertically, directly from parent to offspring, or horizontally, by reacquiring the community from the environment (Bright and Bulgheresi 2010). Despite the transmission of the bacterial symbionts from the ANG to the egg jelly coat, this symbiosis is hypothesized to be environmentally transmitted (Kaufman et al. 1998, Barbieri et al. 2001, Kerwin and Nyholm 2017). Juvenile squid hatch without a reproductive system, thus female hatchling squid lack an ANG, and as a result do not appear to have a physical organ to harbor the symbionts. The embryos are maintained in an apparently sterile yolk sac within the thick chorion membrane, so vertical transmission would have to occur upon hatching, by obtaining bacteria from the JC as the juvenile exits the egg. Bacteria would then have to be stored somewhere within the juvenile in a non-reproductive organ. The majority of OTUs present in the ANG have also been found in the seawater and sediment of the native squid environment (Kerwin and Nyholm 2017), supporting the hypothesis of environmental transmission. This environmental presence could be seeded by adult females during oviposition, could be from the JCs as they break down post-hatching, or could be a free-living state of the ANG bacteria.

The ANG bacterial community is known to be unaffected by maintenance of adult squid in the laboratory (Kerwin and Nyholm 2017), but whether the community is affected by raising juveniles to adulthood in the lab remains unknown. If the community is environmentally transmitted, the lack of the natural Hawaiian seawater bacterial community should affect the bacterial composition of the ANG in raised animals.

The development of the ANG tissue has only been studied in a few cephalopods: in the cuttlefish *Sepia elegans* and *Sepia officinalis*, and in the squids *Loligo vulgaris* (Döring 1908) and *Doryteuthis opalescens* (Kaufman et al. 1998). The study of the two *Sepia* species and one *Loligo* species is based solely on preserved museum specimens. These studies concluded that cephalopod ANGs arise from paired ectodermal epithelium, and that depressions develop which eventually grow into tubules (Döring 1908), observations which are in line with that of a more recent study on *D. opalescens* (Kaufman et al. 1998). In *D. opalescens* the beginning of ANG development was shown to occur as a single layer of epithelial tissue adjacent to the ink sac in an 87-day-old juvenile (Kaufman et al. 1998). This single layer of tissue was composed of invaginations and was coated in cilia and microvilli. The development of the ANG was traced only until day 129 of development, at which point more layers of epithelial tissue had accumulated, deepening the invaginations (Kaufman et al. 1998). At this final stage investigated, the published TEMs appeared to include invaginations which had begun to close off towards their bases, but the majority of the tissue consisted of invaginations, and no true tubules were observed, leaving some doubt as to the remainder of ANG tissue development.

While the development of the ANG organ has been previously examined in other groups of cephalopods, no one has examined ANG development in the bobtail squids. In addition, no one has examined the development of the ANG symbiotic community in any cephalopod, aside

from some morphological observations. In this study, we examine the development of the ANG and the ANG bacterial consortium over the course of *E. scolopes* sexual maturation. We also examine whether raising juvenile squid in the lab to maturity affects the bacterial composition of the ANG.

Methods

Animal collection

Immature *E. scolopes* females of various sizes were collected from Maunalua Bay, Oahu, HI (n=15, 21°26'3.36"N, 157°47'20.78"W), and Kaneohe Bay, Oahu, HI (n=5, KB, 21°16'51.42" N; 57°43'33.07" W). Mantle length (ML) of the squid was recorded, and the internal anatomy was noted upon dissection. Squid under 20mm ML were considered immature, and generally lacked eggs in the mantle cavity. The ANG coloration and morphology was also recorded. Bobtail squid were sacrificed in Oahu, or were transported to Connecticut and briefly maintained in aquaria prior to sacrifice. Animals were anesthetized in 2% ethanol in filter-sterilized seawater (FSSW) prior to sacrifice. Dissected tissues were surface-sterilized through washing, first in 99% ethanol, then in filter-sterilized squid Ringer's solution (FSSR, Collins et al. 2012).

DNA extraction, sequencing, and analysis

ANGs were homogenized in FSSR and underwent differential centrifugation to remove host tissue from the bacterial cells. To pellet the host tissue, the homogenized solution was centrifuged at 100 Xg for five minutes. The supernatant containing the bacterial cells was then removed and centrifuged at 5,000 Xg for five minutes to pellet the bacteria. DNA was extracted from ANGs using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) and a bead-beater (Mini-Beadbeater-16, BioSpec Products, Bartlesville, OK), as in Kerwin and Nyholm (2017).

DNA concentration was determined using the Qubit® dsDNA High Sensitivity assay (ThermoFisher Scientific Inc., Waltham, MA) and averaged 24.5 ng/µl ± 18.1 ng/µl, all samples were greater than 1.8 ng/µl.

Extracted DNA was amplified with barcoded primers (Caporaso et al. 2012) for the V4 region of the 16S rRNA gene, and sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) following previously described protocols (Benjamino et al., 2016; Nelson et al. 2014). Some samples were processed by the UConn Microbial Analysis, Resources and Services facility.

Extraction and PCR controls were processed and sequenced simultaneously with all samples. Less than 1000 sequences/control were obtained in all cases, and the majority of sequences in these controls belonged to a single *Escherichia* OTU. Most other OTUs present in the controls were not present in the ANG samples. Three *Rhodobacteraceae* OTUs also associated with the community were obtained in the controls as well, but accounted for less than 1% of sequences for the control samples. In addition, the presence of *Rhodobacteraceae* in the ANG has been previously established through the use of fluorescence *in situ* hybridization (Collins et al. 2012) and through culture (Chapter 3). No *Verrucomicrobia* OTUs were found in any of the control samples.

Sequencing data were analyzed following established protocols using QIIME (Benjamino et al., 2016, Nelson et al., 2014, Caporaso et al., 2010). Operational taxonomic units (OTUs) were assigned at the 97% identity level using *de novo* methods. QIIME was used to analyze alpha-diversity, but the log2 Shannon Index was converted to a natural log Shannon Index. Alpha diversity was plotted against female mantle length and a linear regression was performed in PRISM. Beta-diversity PCoA plots were created in Excel based on the Bray Curtis metric.

Taxonomic diversity relative abundance was also plotted against female mantle length and a linear regression and nonlinear regression for an exponential model were performed for this analysis as well in PRISM, with the higher R^2 value curve selected. Sequences were compared to sequences from a prior study (Kerwin and Nyholm 2017) accessioned in the European Nucleotide Archive (ENA) under project ID PRJEB14655, as well as to sequences analyzed in Appendix I.

Microscopy

ANGs from 13mm and 16mm mantle length immature animals, and from a >20mm mantle length mature squid, were prepared for transmission electron microscopy as previously described (Kerwin and Nyholm 2017, Collins et al. 2012). Briefly, ANGs were placed in a 2.5% gluteraldehyde, 2% paraformaldehyde fixative solution in a 0.1 M sodium cacodylate buffer with 0.375 M NaCl (to adjust osmolarity), and 0.0015 M $MgCl_2$ and $CaCl_2$, at pH 7.5. Fixed ANGs were washed in a buffer solution lacking the aldehyde fixatives but otherwise of the same formula as noted above. ANGs were placed in a solution of 1% osmium and 0.8% potassium ferricyanide (for brighter staining of membranes and bacteria) for 1.5 hours at 4° C. ANGs were transferred to a transition fluid, propylene oxide, for two washes of 15 minutes apiece. Propylene oxide was used when ethanol was found to not provide for effective embeddment. ANGs were embedded in Spurr's epoxy resin. Embedded ANGs were sectioned on a Leica UCT Ultramicrotome. Semi-thin sections (1 μm thick) were cut with a glass knife, stained with a 1:1 solution of Azure II and Methylene Blue, and visualized via a Zeiss Axiovert 200M light microscope to examine gross morphology. Ultrathin sections (90 nm thick) were generated using a diamond knife and were collected on thin-bar 300 count grids. Sections were stained with uranyl acetate for ten minutes, and with lead citrate for five minutes. All samples were imaged

on a FEI Technai Biotwin transmission electron microscope at a variety of magnifications. For all samples a minimum of three sections were observed, and for most samples 7-12 sections were observed.

Raising experiments

Hatchling squid were obtained from eggs deposited by laboratory-maintained, wild-caught *E. scolopes*. Hatchlings were raised to adulthood on a diet of mysid shrimp followed by glass shrimp, in aerated water which was either 0.2µm filtered and changed frequently, or was flowing and UV-irradiated, with autoclaved sand. Raised animals were transferred to aquaria used to house adult squid once they reached 10-15mm mantle length. Squid were sacrificed after reaching adult size and ANG bacterial DNA was extracted and sequenced as described above.

Additional raised animal ANGs were obtained from collaborators at the University of Wisconsin-Madison. These animals were raised under similar conditions (Koch et al. 2013), but the artificial seawater was not filter-sterilized, and the sand provided was originally from Oahu, Hawaii.

Hatchling squid microbiome

Juvenile squid which had hatched within the past 48 hours were collected and transferred to FSSW (n=5). Animals were anesthetized in 2% ethanol in FSSW and then immediately flash-frozen. The entire juvenile was homogenized with a plastic pestle in FSSR. Homogenized tissues were processed in the same manner described for ANGs above.

Results

ANG tissue development

While *E. scolopes* mantle size (distance from base of eyes to posterior of squid) is only loosely correlated with age under laboratory conditions (personal obs.), mantle size does appear

to be correlated with maturity level (Figure 1B). Females under 10mm mantle length (ML) have never been found with visible ANGs or nidamental glands, and squid of that size are generally quite difficult to sex. When squid reach 10-13mm ML these organs were present but quite small. At the macroscopic level, the smallest animals (10-13 mm ML) had two small nidamental glands which lacked the brightly colored channel found in adults, and contained a transparent tri-lobed structure, presumed here to be the nascent ANG, each wing of which was covered in regular pits, with the edges containing channels of some sort (Figure 1C).

In larger animals (13-15mm ML), the ANG appeared to assume a bi-lobed structure which was a fairly translucent white/cream color, although more opaque than the previous stage, and was still quite small (Figure 1C). By the time females approached 15 mm ML, the bi-lobed structure appeared merged into a single tissue, vaguely heart-shaped, similar to that found in adults, but still quite small (Figure 1B). Tubules may or may not have been distinguishable at this stage, but the organ was opaque and usually a beige/white color. In still larger animals (15-17mm ML), the ANG had begun to resemble that of the adult more and more, with tubules that were generally white or beige with only a few spots of color, generally yellow or red (Figure 1C). In females with a ML of 18-20mm the ANG appeared very similar to that of mature animals, more pigmented and dense tubules composing the entire volume. A couple of eggs were occasionally present in the mantle cavity at this point, and the nidamental glands were fully formed with the pink channel that runs down the center of each present (Figure 1B). By 20mm females were almost always completely mature, with a mantle cavity full of eggs (Figure 1C).

Microscopically, changes in the structure of the ANG were also observed as females matured. At the stage in which the ANG had a bilobed structure (Figure 1C, 13mm ML), the ANG surface contained numerous ciliated invaginations (Figure 2). Few to no bacterial cells

were present at the surface of these shallow invaginations (Figure 2A, 2E, 2F), although a few bacteria, as well as other debris, were observed at the base, or in the crypt, of some of the invaginations extending deeper into the tissue (Figure 2G). The ANG tissue at this stage also appeared to be divided into two distinct layers (Figure 2C, 2E). The surface layer contained the invaginations, some of which extended into the basal layer which contained the tubules. The surface layer also appeared to lack vascularization, while blood vessels were common in the basal layer. One image (Figure 2B) captured the region of the ANG immediately adjoining the nidamental glands, and appeared to be a cross section of a field of ciliated invaginations. At this size (13mm ML), the ANG already had a multitude of tubules, although most of these tubules were quite small (Figure 2B, 2C, 2D). The majority of the tubules examined contained coccoid-shaped bacteria, many of which were in the process of cell division (Figure 2F), although some rod-shaped bacteria were also observed (Figure 2G). The surface of this organ was covered in a thick brush border of microvilli (Figure 2A, 2F) and cilia (Figure 2F).

An ANG from a larger immature female (Figure 1E, 16mm ML), while still appearing fairly immature macroscopically, resembled the mature ANG much more closely (Figure 3). The tissue in this ANG was uniform, composed throughout of larger tubules with dense bacteria (Figure 3A-C). No ciliated invaginations were found on the surface of this ANG, and the surface also lacked microvilli and cilia. The surface of a mature ANG (>20mm ML) also lacked any ciliated invaginations, and the tissue was also uniform without a surface layer (Figure 3E-F). Vascularization of both this and the 16mm ANG was also noted.

In an attempt to quantify some of the variation seen between the tubules from the 13mm, 16mm, and mature ANG, the width of the epithelial layer for 35 tubules/ANG was measured (Figure 3). Epithelial width was chosen over tubule diameter as tubule diameter varied widely

depending on the angle at which the tubule was sectioned. The width of the 13mm tubule epithelium was variable, reflecting the varying stages of tubule development found in that tissue, and averaged 5.6 μm ± 1.6 μm, while that of the 16mm and mature ANG were less variable and quite similar to each other (3.7 μm ± 1.0 μm, and 3.5 μm ± 0.9 μm respectively). The tubule epithelium of the smallest size class was significantly wider than either of the other size classes ($F_{2,99} = 35.35$, $p < 0.0001$).

ANG bacterial community development

To examine potential shifts in the bacterial community over the course of ANG development, a wide range of ANGs from variously sized immature and mature females were examined (10.5-30mm ML). All ANGs contained the same four dominant bacterial classes: *Alphaproteobacteria*, *Opitutae* (a class of *Verrucomicrobia*), *Gammaproteobacteria*, and *Flavobacteriia* (Figure 5A). However, the relative abundances of these classes shifted as the ANG matured. A negative exponential relationship between mantle length and *Opitutae* abundance ($R^2 = 43.1\%$, Figure 5B) and a corresponding positive exponential relationship between mantle length and *Alphaproteobacteria* ($R^2 = 26.1\%$, Figure 5C) were found. Both of these curves began to level off around the 18-20 mm ML mark. *Flavobacteriia* (Figure 5D) and *Gammaproteobacteria* (Figure 5E) abundance were both exhibited a positive linear correlation with mantle length ($R^2 = 18.1\%$, $p = 0.004$ and $R^2 = 5.9\%$, $p = 0.11$ respectively), although only the *Flavobacteriia* slope was significantly greater than zero. The abundance of additional taxa, while always low, decreased exponentially with mantle length, leveling off by a size of approximately 16.5 mm ($R^2 = 13.1\%$, Figure 5F).

The alpha diversity of the ANG bacterial community was also positively correlated with squid size, although the effect varied by metric. The Shannon Index, a measure of community

richness and evenness, significantly increased with squid size ($R^2 = 22.7\%$, $p < 0.001$, Figure 6A). However, neither Chao1 (Figure 6B), a measure of richness which utilizes rare OTU abundance, nor Phylogenetic Diversity (Figure 6C), a measure of how phylogenetically diverse a community is, had a slope significantly greater than zero ($R^2 = 7.1\%$, $p = 0.08$ and $R^2 = 5.6\%$, $p = 0.12$ respectively), indicating that by these metrics alpha diversity is fairly stable across size classes. As richness did not significantly increase with size (Chao1), the significant increase in the Shannon Index can be attributed to an increase in community evenness.

A Bray Curtis beta diversity analysis of ANG communities associated with various maturity levels of *E. scolopes* females did not result in an entirely clear cluster pattern linked to size (Figure 7). While most of the smaller animal (< 18mm mantle length) ANGs clustered towards one side of the PCoA plot, the cluster was not especially tight and included some larger squid samples as well, and was thus not entirely distinct from the more mature communities. Once squid approach maturity, size no longer appeared to correlate with community composition, perhaps reflecting variability in maturity levels in these size classes.

ANG development in laboratory-raised animals

In an effort to directly correlate squid age with ANG development, juvenile squid were raised to adulthood. Laboratory-raised squid frequently did not develop an ANG at all, and those that did had an ANG that appeared to lack much color, and to be stunted and asymmetrical (Figure 8, Collins 2014). The McFall-Ngai lab, while at the University of Wisconsin, also raised juveniles to adulthood. These females consistently had ANGs, which tended to be symmetrical and to be more colorful than those from our lab, although still stunted slightly compared to wild ANGs (pers. obs.). ANGs from females that did develop an ANG in our lab (hereafter referred to

as CT-raised) and ANGs from lab-raised females produced by the McFall-Ngai lab (hereafter referred to as WI-raised) were dissected and analyzed for bacterial community composition.

The bacterial communities of adult CT-raised (n=4) and WI-raised (n=9) female ANGs were quite variable. The average community of CT-raised ANGs was composed of *Alphaproteobacteria* and *Flavobacteriia*, and lacked both *Verrucomicrobia* and *Gammaproteobacteria*, while the WI-raised ANG was composed almost exclusively of *Alphaproteobacteria* (Figure 9A). However, both of these communities were highly variable, as can be seen when looking at the families present. For example, while all raised ANG communities contained members of the *Rhodobacteraceae*, in two of the CT-raised communities these OTUs accounted for less than 0.1% of the community, while in a third CT-raised ANG these OTUs accounted for 73% of the community. The WI-raised ANG community was more consistent, with seven ANGs containing between 96% and 99.9% *Rhodobacteraceae* OTUs. However, the other two WI-raised ANGs contained 81% and 31% *Rhodobacteraceae* respectively, demonstrating that these communities were variable as well. The presence of other *Alphaproteobacteria* families was equally variable (Figure 9B). *Verrucomicrobia* were found in one CT-raised ANG (8.1%) and one WI-raised ANG (5.8%) at substantial concentrations, although low levels (< 0.03%) were also found in the other three CT-raised and five additional WI-raised ANGs (Figure 9E). *Flavobacteriia* were found exclusively in CT-raised ANGs, but were highly variable in abundance (Figure 9D), while *Gammaproteobacteria* were only found at high levels in two WI-raised animals (Figure 9C). *Gammaproteobacteria* were also present at very low levels (< 0.02%) in two CT-raised ANGs and two additional WI-raised animals.

The alpha diversity of both CT- and WI-raised ANGs was lower than that of wild ANGs of similar mantle lengths, falling well below the linear regression curve discussed above for the

varying developmental stages (Figure 10A-C). The lower alpha diversity of raised ANGs was significantly lower than that of wild adult ANGs for all three metrics examined: Shannon Index ($F_{3,54} = 24.74$, $p<0.0001$), Chao1 ($F_{3,54} = 10.91$, $p<0.0001$), and Phylogenetic Diversity ($F_{3,54} = 16.41$, $p<0.0001$, Figure 10D-E). The WI-raised ANGs were also significantly lower than that of immature wild ANGs for both the Shannon Index and Phylogenetic Diversity, although not for Chao1 (Figure 10D-E).

A Bray Curtis beta diversity analysis of the raised animal ANGs demonstrates the importance of *Verrucomicrobia* abundance in defining the ANG community. WI-raised ANGs form a tight cluster apart from both the CT-raised and wild ANG samples (Figure 11). The one WI-raised ANG that is not part of this cluster is the sole WI-raised ANG that contains *Verrucomicrobia* at a substantive level (5.8%), while similarly the one CT-raised ANG that clusters with the wild ANGs is also the sole CT-raised ANG that contains 8.1% *Verrucomicrobia*. This analysis also demonstrates that while macroscopically the ANGs in WI-raised females may appear more similar to wild ANGs, the bacterial composition of CT-raised ANGs is actually more similar to wild ANGs, probably due to the presence of *Flavobacteriia* in these communities.

To more closely examine the potential for vertical transmission of the ANG symbiosis through inoculation from the jelly coat community, we sequenced the microbiome of the entire hatchling squid after one to two days of hatching. For all five juveniles examined the dominant OTU (89%-99.5% of the community) was an *Allivibrio*, probably indicating the established light organ colonization by *V. fischeri*. In regards to ANG community members, one *Rhodobacteraceae* OTU which is common in mature ANGs accounted for an average of 0.23% of the community (maximum of 0.6%). One juvenile had the *Verrucomicrobia* OTU which

accounts for 25% of the mature ANG community on average, at the low abundance of 0.04%, but this OTU was completely lacking from the other four juveniles examined. Overall this analysis does not appear to provide support for vertical transmission via hatching, but further analysis with more juveniles and at different developmental stages is needed.

Discussion

Our macroscopic and microscopic observations of various stages of immature *E. scolopes* ANGs has led us to propose the following progression for development (Figure 1). As the reproductive system begins to develop in immature females, the nidamental glands form first. A thin epithelial layer arises at the base of the nidamental glands which forms into a tri-lobed ciliated field covered in microvilli and ciliated invaginations which can sample bacteria as seawater is flushed through the mantle cavity (Figure 1A). The cilia within the invaginations could be a mechanism to push the captured bacteria deep into the invaginations, or, similar to what has been described for the *E. scolopes* light organ symbiosis, could actually be preventing the entry of non-symbiont bacteria into the invaginations (Nyholm and McFall-Ngai 2004). Either way, the dense assortment of cilia could explain the lack of bacterial cells at the superficial end of these invaginations. As bacteria enter the invaginations and colonize the base or crypts, the invaginations close off into tubules, and a new epithelial layer forms on top of that layer with new invaginations (Figure 1A). Alternatively, the invagination crypts could bud off after colonization occurs, and the newly empty invagination could grow outwards as new epithelial cells form (Figure 1A). Once sufficient colonization has occurred the ciliated invaginations all close off into tubules, or possibly the superficial layer of tissue is shed, and the surface of the ANG loses its microvilli and cilia, although those are maintained in the tubules

themselves (Figure 1A). As the bacterial colonizers divide and multiply, the tubule lumen expands, compressing the epithelial cells which line the tubules.

The primary differences between the immature and mature ANG appears to be the presence of microvilli and cilia covering the surface of the immature ANG, but lacking from the mature ANG, and the presence of two tissue layers in the immature ANG, with the superficial layer containing ciliated invaginations, and the vascularized basal layer containing tubules (Figures 2-3). Examination here of a larger immature ANG, as well as of the surface of a mature ANG, has revealed that the ciliated epithelial layer present at the beginning of ANG tissue development is not maintained in mature squid. The time necessary for an animal to grow from 13mm mantle length to 16mm mantle length is not long, probably only 1-1.5 weeks if food is abundant (data not shown), so these invaginations appear to only be present long enough to presumably allow for initial colonization. The structure of the 16mm mantle length immature ANG was actually strikingly similar to that of the mature ANG (Figure 3), surprising given the different macroscopic appearances, and the different bacterial community compositions described here. These results indicate that by this stage colonization of the ANG is complete and the tissue has reached its mature structure, although the distinct bacterial community and lack of coloration/smaller size of the organ demonstrate that within the ANG the community is still growing towards its equilibrium, which will continue to increase the size of the tubules as the squid matures. In the future, this hypothesis could be tested in part by examining the bacterial load of immature ANGs.

While prior research found a strong correlation between *E. scolopes* size and age (Hanlon et al. 1997), our experiences raising juvenile *E. scolopes* has shown that similarly aged squid can have widely varying mantle lengths (data not shown). Despite the strong linear correlation

reported by Hanlon and colleagues (1997), an examination of their data points shows that such variation was also found in their systems, with, for example, 60 day old animals ranging from 8-16mm ML. However, the data reported here demonstrates that female *E. scolopes* ML does correspond with maturity, and specifically with reproductive system development.

The developmental progression of the ANG as described here presents striking similarities to that of the development of the ANG from another cephalopod, the myopsid squid *D. opalescens*, and the development of the *E. scolopes* light organ. Both of these symbiotic organs form as epithelial layers adjacent to the ink sac (Montgomery and McFall-Ngai 1993, McFall-Ngai and Ruby 1991, Kaufmann et al. 1998), while the *E. scolopes* ANG forms as an epithelial layer adjacent to the nidamental glands, slightly posterior to the ink sac. The nascent light organ is composed of a superficial ciliated epithelium that undergoes apoptosis and regression in response to the light organ symbiont *V. fischeri* (Montgomery and McFall-Ngai 1993, McFall-Ngai and Ruby 1991). Analogously, the ANG tubules may form as invaginations from a ciliated surface epithelium (Kaufmann et al. 1998, this work). The light organ forms ciliated fields that are shaped like appendages to facilitate colonization, and develop by extension and growth of the surface epithelium (Montgomery and McFall-Ngai 1993), similar in some ways to the ciliated field that we see here for the ANG. In the future, examining the biomechanics of the ANG field could provide further insight. The light organ ciliated appendage cells each have cilia surrounded by microvilli, similar again to the immature ANG surface, although the cilia on the ANG appear to be shorter than those on the light organ ciliated appendages (~10 μm vs 20 μm respectively), as are the microvilli (~1μm vs 1-2μm respectively, Montgomery and McFall-Ngai 1993). The light organ ciliated appendages are gradually lost, starting within 24 hours of crypt colonization, and completely disappear within three days of

colonization, although they are retained in uncolonized animals (McFall-Ngai and Ruby 1991, Nyholm and McFall-Ngai 2004). Similarly, the results of this work suggest that the microvilli and ciliated surface of the ANG disappears as tubules form and take over, although both structures persist within the tubules themselves. We hypothesize that the superficial epithelial layer containing the ciliated invaginations may be shed as part of morphogenesis, although more research is necessary to examine this possibility further.

Although it remains to be tested, ANG bacteria may induce similar morphological changes as described with the light organ symbiosis. Future research should test whether certain ANG bacteria induce tubule development and under what circumstances tubules will or will not form. It may be that the ciliated field of the ANG eventually regresses leaving no sign of this superficial structure (Figure 8). In the case of the ANG with its much more complex community compared to that of the binary light organ symbiosis, the full community may be necessary to form a full and symmetrical ANG. Therefore, when animals are raised in the lab where certain community members are lacking, only some tubules may develop, explaining the asymmetrical and stunted ANGs present in certain lab-raised squid, as well as their lower alpha diversity and dominance by *Alphaproteobacteria*, which tend to be present in laboratory environments (Figures 8-10, Kerwin and Nyholm 2017). In the future, dissecting raised animals at mid-stages of development, around 10-14 mm mantle lengths, should help to answer some of these questions, and provide further proof that formation of a normal ANG community should be possible if the correct community is provided at the right level of abundance.

To our knowledge, no one has described the community composition of the ANG for any cephalopod over the course of development. The changes found in community alpha diversity together indicated an increase in evenness, as a community dominated by only one type of

bacteria shifted to a community with several different members all making up a substantial part of the community (Figures 5-6). Community composition as a whole shifted slightly between immature and mature squid, but no distinct patterns were found by the time squid reached a mantle length of 18mm, indicating that the bacterial community was mature by this stage, even if the entire reproductive system may not be (Figure 7). The striking change from a *Verrucomicrobia*-dominated community to an *Alphaproteobacteria*-dominated community characterized here through next-generation Illumina sequencing was supported by our microscopy, in which most of the tubules found in the immature ANG contained coccoid-shaped bacteria (Figures 2 and 5). Previous work from our lab has hypothesized that the cocci in the mature ANG are the *Verrucomicrobia*, while the *Alphaproteobacteria* are rod-shaped (Collins et al. 2012).

The ciliated field of the ANG appeared to persist for only a short time, probably less than two weeks, thus any patterns of bacterial succession would have to be established early. Later shifts in community composition would therefore have to be explained by mechanisms other than new bacterial colonization. If community composition shifts were due entirely to bacterial colonization patterns, we would expect to find tubules containing *Verrucomicrobia* towards the center of the ANG, with the *Alphaproteobacteria* outside of those, but prior research has not found any patterns to bacterial tubule persistence (Collins et al. 2012). Additionally, even in the smallest ANG sequenced to date, a 10.5mm mantle length squid, all of the bacterial taxa found in the mature ANG were present (Figure 5). The *Verrucomicrobia* may be better colonizers of the early ANG, reflecting their early dominance. It should also be noted that all estimates of abundance here are relative, and do not reflect absolute abundance – the bacterial load of the early ANG is almost certainly substantially lower than the 10^{10} bacteria/ANG load associated

with the mature ANG (Collins et al. 2012). The exponential shift in abundance may then reflect the very different growth rates of *Verrucomicrobia* and *Alphaproteobacteria*. *Verrucomicrobia* are known to have very slow doubling times, taking a month or more to form visible colonies on agar plates (Stevenson et al. 2004). While the *Verrucomicrobia* species present in the ANG has not yet been cultured, we have no reason to suspect that this species grows at a much faster rate, although microconditions of the tubules are unknown, and may foster growth for certain taxa. The ANG *Alphaproteobacteria* however, are known to be dominated by the genus *Leisingera*, and most cultured isolates of *Leisingera* from the ANG have a doubling time of only four hours under most conditions (A. Suria, pers. comm.) Thus, one potential explanation of the relative abundance shifts over development could be that while the *Verrucomicrobia* are the initial dominant colonizers, once the *Leisingera* colonize tubules their much faster doubling time causes the *Alphaproteobacteria* tubules to grow much more rapidly, quickly overtaking the *Verrucomicrobia* tubules in terms of volume.

Alternatively, the various tubules may also change once colonized by a specific bacterial species to provide a microenvironment that fosters the growth of that species. Hemocytes are known to traffic into the ANG tubules (Collins et al. 2012), similar to what has been shown in the light organ (Nyholm and McFall-Ngai 1998, Schwartzmann et al. 2015). Cephalopod blood contains hemocyanin, which is similar to hemoglobin in that it follows the Bohr effect: the oxygen binding affinity is inversely correlated with acidity and carbon dioxide concentration (Kremer et al. 2014). When hemocytes traffic into ANG tubules they could affect oxygen concentrations. While *Leisingera* grow well under aerobic conditions, *Verrucomicrobia* have generally been found to grow best under anaerobic, microaerophilic or enhanced carbon dioxide (Stevenson et al. 2004), thus these two main colonizers of the ANG appear to have different

oxygen needs. Hemocytes are known to contribute chitin to *V. fischeri*, by trafficking into the crypts, lysing, and releasing chitin granules (Schwartzmann et al. 2015). The light organ symbiont, *V. fischeri*, can catabolise chitin, and thus acidify the light organ crypts at night (Schwartzmann 2015). The acidification of the crypts allows for greater release of oxygen from hemocyanin which supports luminescence (Kremer et al. 2014). The number of hemocytes necessary to acidify the light organ crypts has been estimated to be approximately 2000, or about 5% of the circulating hemocyte population. Given that the light organ is only a small fraction of the volume of the mature ANG, the ANG tubules are unlikely to be acidified to a similar extent, however, hemocytes could be influencing the tubule microenvironment in subtler ways, enough to create varying environments that aid in the growth of the different bacteria. Additionally, other aspects of the host physiology, such as the varying brush borders which line different tubules, and the presence of secretory vesicles, could work together with the host immune system to form varying microenvironments that promote the growth of different bacterial symbionts.

Previous research has noted the similarities between the ANG and light organ, and proposed that the light organ may have potentially evolved as a specialized version of the ANG, containing only luminous bacteria, and displacing the ANG to a posterior position, although this hypothesis is rather speculative (Montgomery and McFall-Ngai 1993, Kaufmann et al. 1998, Nishiguchi et al. 2004). This hypothesis may explain why cephalopods that don't have a light organ have an ANG that is closely associated with the ink sac, as is seen in *D. opalescens* (Kaufmann 1998). Females from another sepiolid genus, *Semirossia*, sometimes have a duct which connects their light organ to the ANG, rather than the light organ venting directly into the mantle cavity (Boletzky 1970). Prior research into the development of the ANG tissue described for the Pacific market squid, *D. opalescens*, only characterized the early stages of development,

leading up to the very beginning of tubule formation (Kaufmann et al. 1998), but those stages appear to fit in with our macroscopic observations, and to dovetail with our microscopic observations, indicating that ANG development is probably similar in mysopsids and sepiolids, despite their fairly different bacterial community composition (Appendix II).

The presence of microvilli is frequently associated with tissue which is poised for environmental, or horizontal, symbiont colonization. *Bathymodiolus* sp. mussel gills are competent throughout the life of the mussel, but the gill tissues are colonized in a continuous process as those tissues develop (Wentrup et al. 2014). The youngest gill filaments are coated in microvilli on their surface to aid in colonization by their symbionts, then once partial colonization has occurred the presence of microvilli is substantially reduced, and fully colonized gill filaments lack microvilli entirely (Wentrup et al. 2014). The microvilli coated surface of the immature ANG and presence of the ciliated invaginations, along with the varying community composition of laboratory-raised animals, provides further evidence that the ANG symbiosis is reacquired from the environment each generation. The one potential source of vertical transmission could be as the hatchling squid passes through the jelly coat of its egg capsule, which contains the ANG bacterial community (Kerwin and Nyholm 2017). The bacterial community could then be carried elsewhere in the squid until the ANG is competent for colonization. However, our investigation of the juvenile microbiome does not support this hypothesis, although further research is needed at different juvenile stages. Unfortunately, the light organs of the juveniles selected for sequencing had been colonized by *V. fischeri*, providing an overwhelming signal of *Allivibrio*, which may have swamped the signal of other, less abundant microbes. Only one of the juveniles contained the *Verrucomicrobia* OTU associated with the adult ANG community, while all contained the dominant *Alphaproteobacteria* OTU. In

the future additional sequencing of juveniles with uncolonized light organs, collected immediately after hatching, may help to further answer this question.

In this study, we examined the development of the ANG tissue, the development of the ANG bacterial community, and how that ANG community varies when squid are raised in a laboratory setting. Our findings of the organ development process link the ANG development of sepiolid squid to that of the myopsid squid *D. opalescens*. The use of superficial ciliated epithelia in the nascent ANG and light organ may indicate similar host colonization strategies are used for recruiting environmental bacteria. Shifts identified in the ANG bacterial community over development provide the basis for interesting insight into the succession of a complex bacterial community, while differences between the ANG bacterial composition of raised and wild animals provides further evidence for environmental transmission of this symbiosis. Future work will focus on examining raised females at various points of development to confirm whether the ciliated field appears in raised animals and regresses or disappears completely without the presence of ANG symbionts in the environment. Additionally, future raising experiments will expose developing hosts to ANG symbionts at environmentally relevant concentrations at the time when the ciliated field is present and thus attempt to induce full ANG morphogenesis.

The development of complex, non-digestive, bacterial symbioses in a maturing multicellular host remains poorly understood. Recent research on bacterial succession in cheese rinds has demonstrated that given specific environmental conditions and bacterial seed species, bacterial succession is replicable (Wolfe et al. 2014). Another study on the hindgut bacterial community of the termite, *Reticulitermes flavipes*, found that an artificial neural network can be trained to predict the outcome of bacterial interactions and show succession of the hindgut community when termites are fed various diets (Benjamino et al. in prep). Here we demonstrate

that the *E. scolopes* ANG bacterial community has a predictable succession, and can be used as a model for understanding interactions of host tissue with a complex bacterial community to select and structure a consortium with important functional implications.

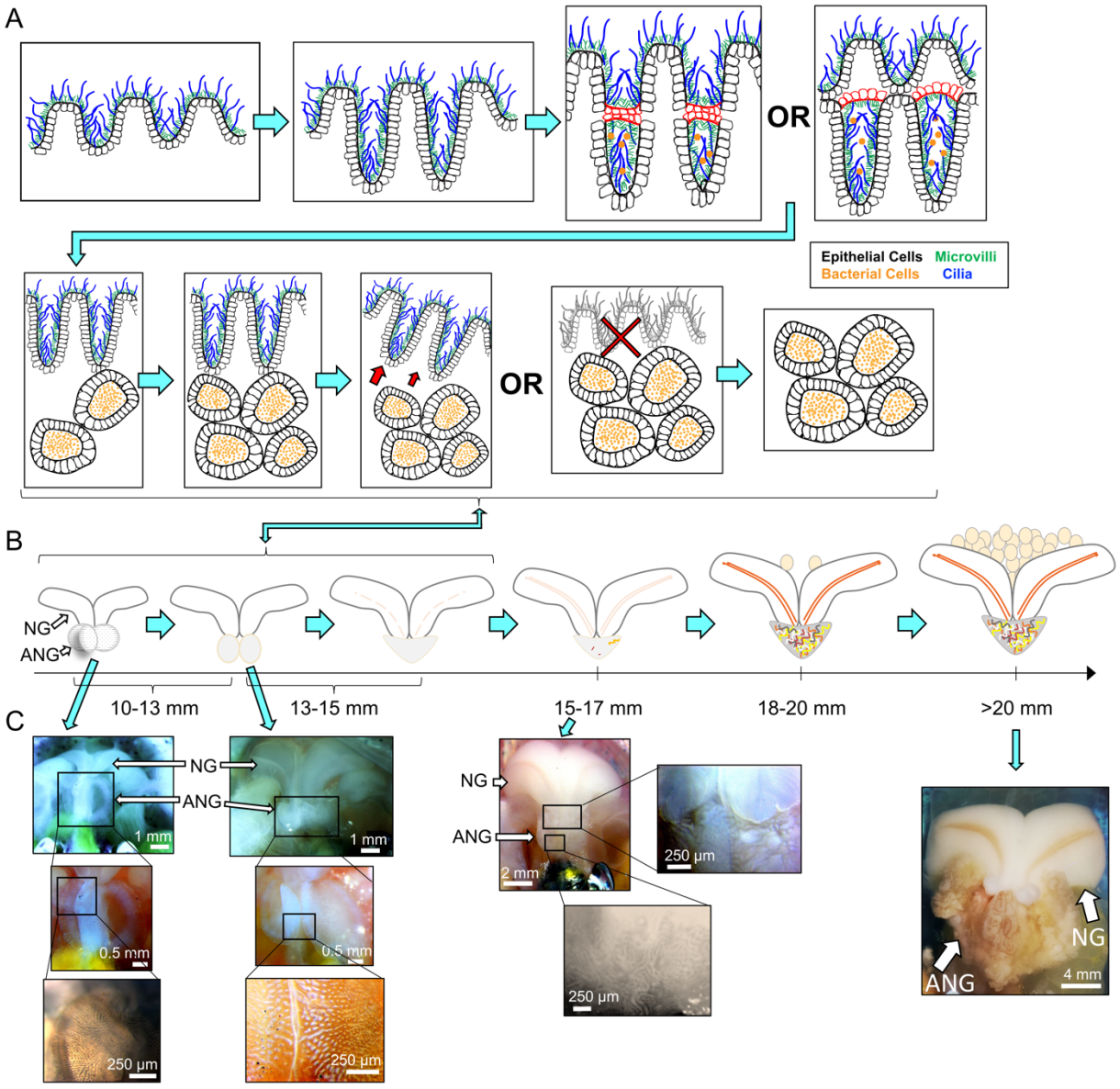


Figure 1: Diagram illustrating the macroscopic developmental changes to the ANG as female *Euprymna scolopes* mature. The nascent accessory nidamental gland (ANG) appeared to form as a transparent tri-lobed ciliated field adjacent to the nidamental glands (NG), which collapsed into a bi-lobed structure which was opaque, and then merged to form a single, heart-shaped tissue which still lacked the coloration associated with the adult ANG (B). As the NG channels became more apparent, sporadic tubule coloration was noted, and by the time squid reached the 18-20mm ML size the ANG and NG appeared to strongly resemble that of adult squid, although animals still lacked many eggs in their mantle cavity (B). Size ranges provided are estimates, and ANG development stages may form slightly earlier or later than indicated in different individuals. A potential model of ANG tissue development is provided for the early stages of development (A), in which new epithelial tissue growth is in red. Macroscopic images of four stages of ANG development are also provided (C).

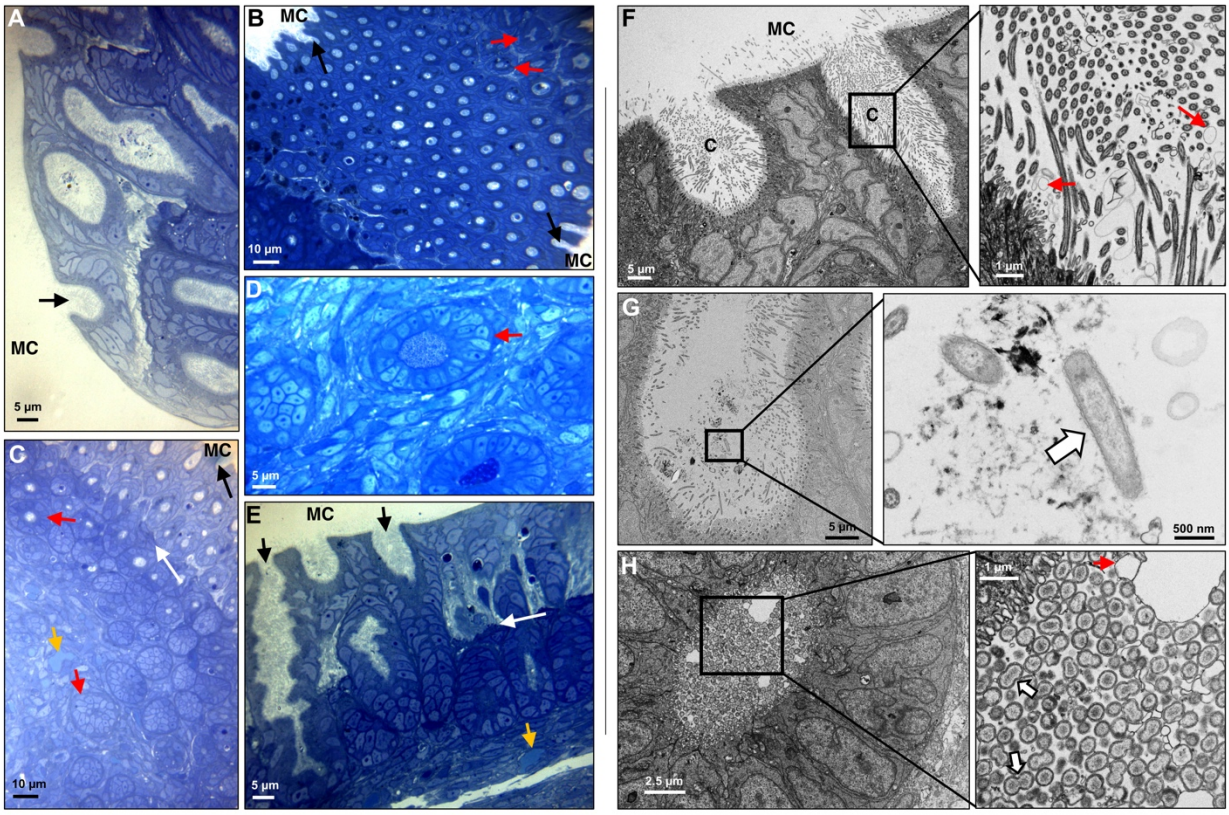


Figure 2: ANG sections from an immature, 13 mm mantle length female. Ciliated invaginations (black arrows, A,B,C,E) can be seen on the surface of the tissue leading from the mantle cavity into the tissue. Small tubules have formed in the interior of the tissue and some contain bacterial cells (red arrows, B,C,D). Two layers of tissue are present, with the top layer consisting of the invaginations and the deeper level consisting of tubules (white arrows show division, C,E). Vascularization was noted only in the deeper level (yellow arrows, C, E). Transmission electron micrographs show the surface (F) and crypt (G) of ciliated invaginations, and a tubule containing dividing bacteria (white arrows, H), in more detail, with secretory vesicles present in both (red arrows). MC = mantle cavity, C = cilia.

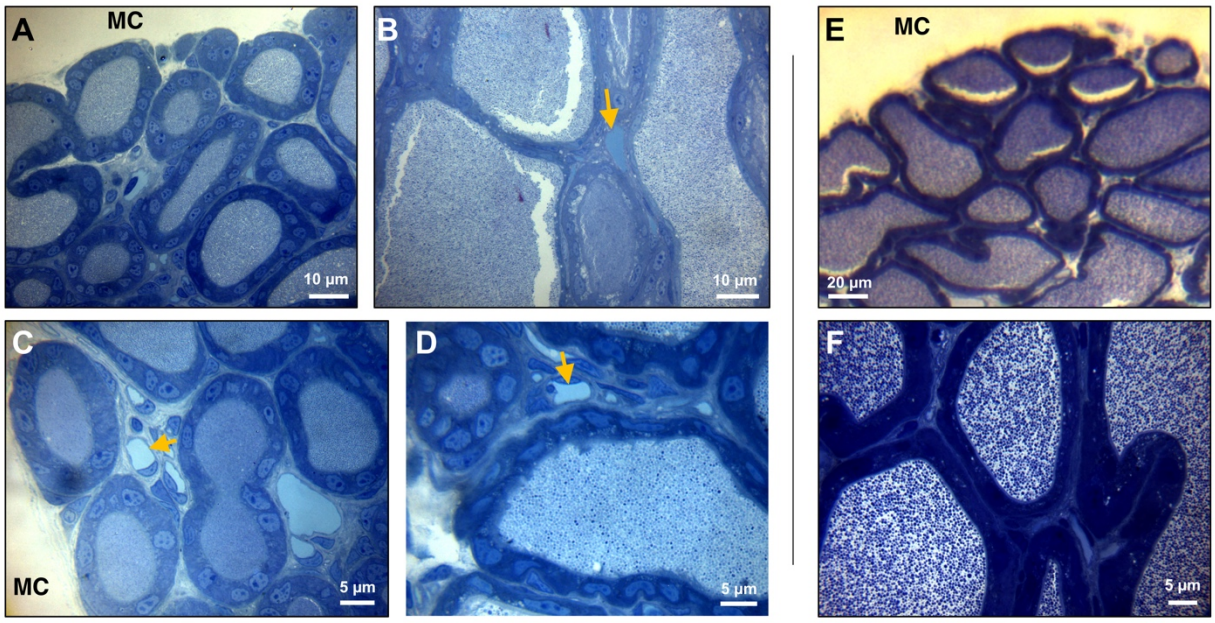


Figure 3: ANG sections from an immature, 16 mm mantle length female (A-D), resemble those of a mature, >20 mm mantle length female (E-F). No ciliated invaginations were found in this still immature but larger female, and the tissue did not contain different layers, but was instead consistently composed of bacteria-filled tubules. Yellow arrows = blood vessels, MC=mantle cavity.

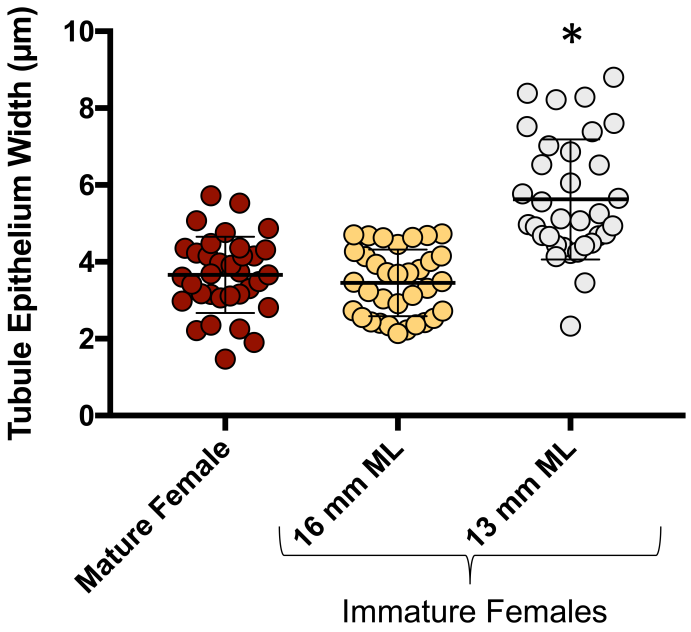


Figure 4: Tubule epithelium width of a 13mm mantle length (ML) immature female was significantly wider than that of a 16 mm ML immature female and a mature female ($F_{2,99}$ = 35.35, p<0.0001).

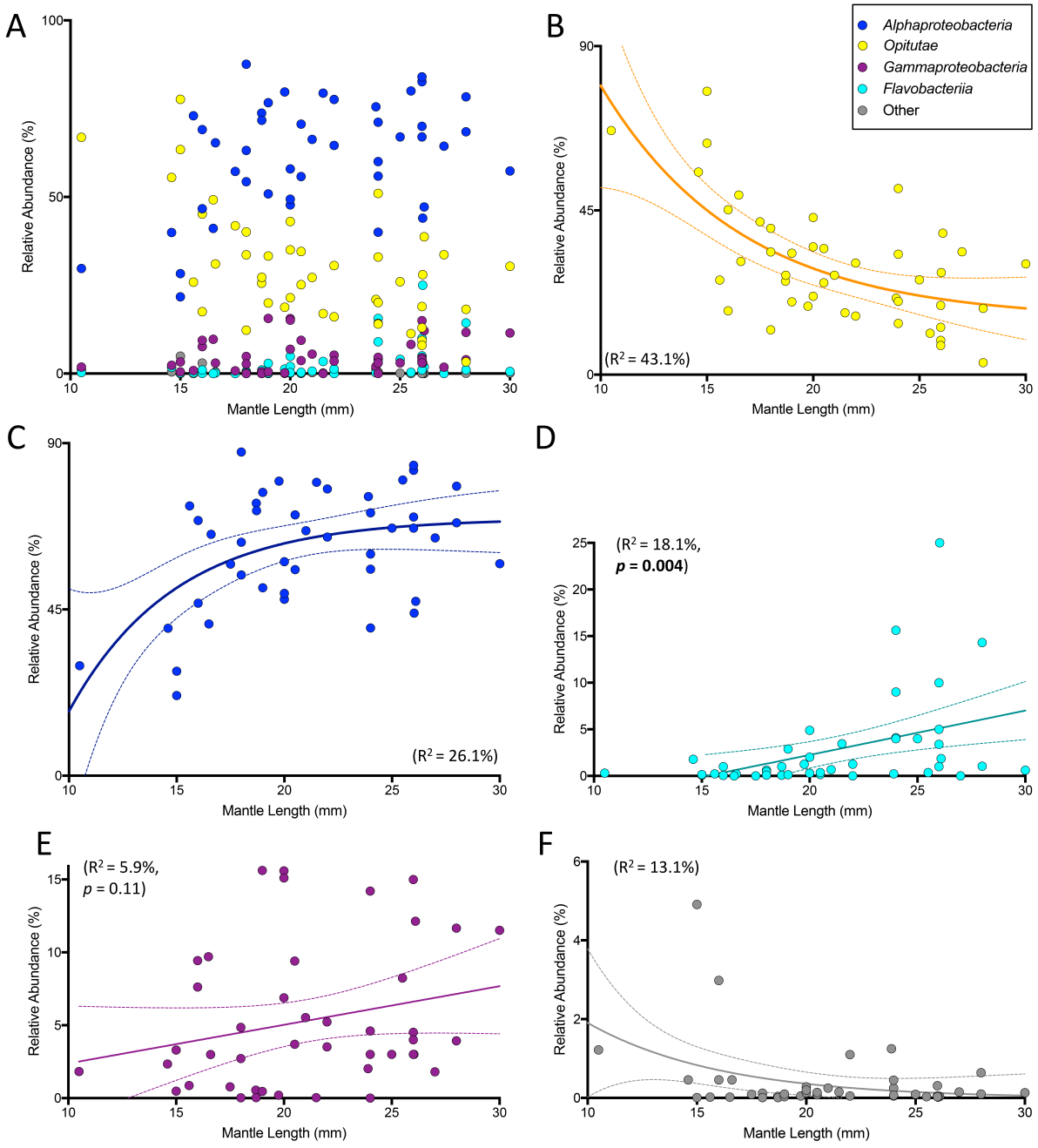


Figure 5: Relative abundances of the bacterial taxa found in the ANG shifted as squid mature. At all maturity stages the ANG consisted of four main bacterial taxa (A). The *Opitutae* (class of *Verrucomicrobia*) decreased (B) while the *Alphaproteobacteria* increased **(**C) with squid size, both exponentially, with curves leveling off around the 20mm size that is associated with adulthood. The *Flavobacteriia* (D) and *Gammaproteobacteria* (E) both increased with squid size in a linear manner, while the relative abundance of other taxa, while always low, decreased exponentially (F), with the curve leveling off even earlier, at approximately 17-18mm mantle length. Note varying y-axes. R^2 value describes goodness-of-fit to exponential curve (B, C, F) or linear curve (D, E), while p-value describes whether linear curve is significantly different from a slope of 0 (D, E).

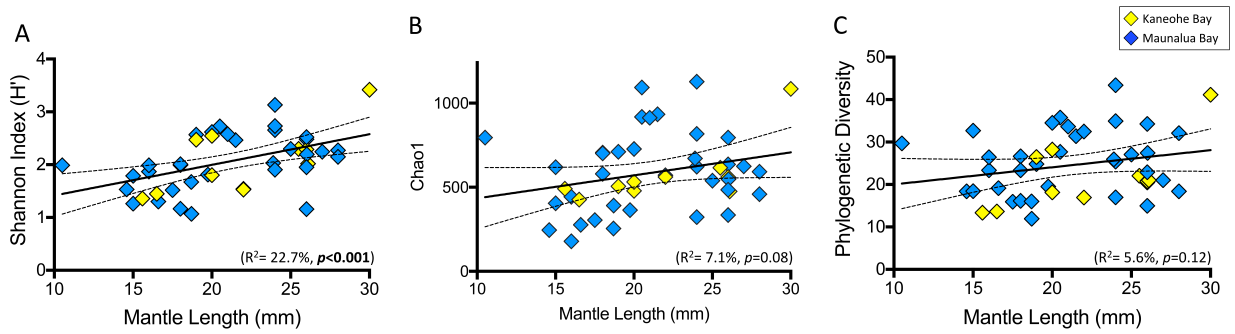


Figure 6: Various measures of alpha diversity show an upward linear trend of diversity as squid mature. However, only the Shannon Index (A) shows a slope significantly higher than 0, while Chao1 (B) and Phylogenetic Diversity (C) do not.

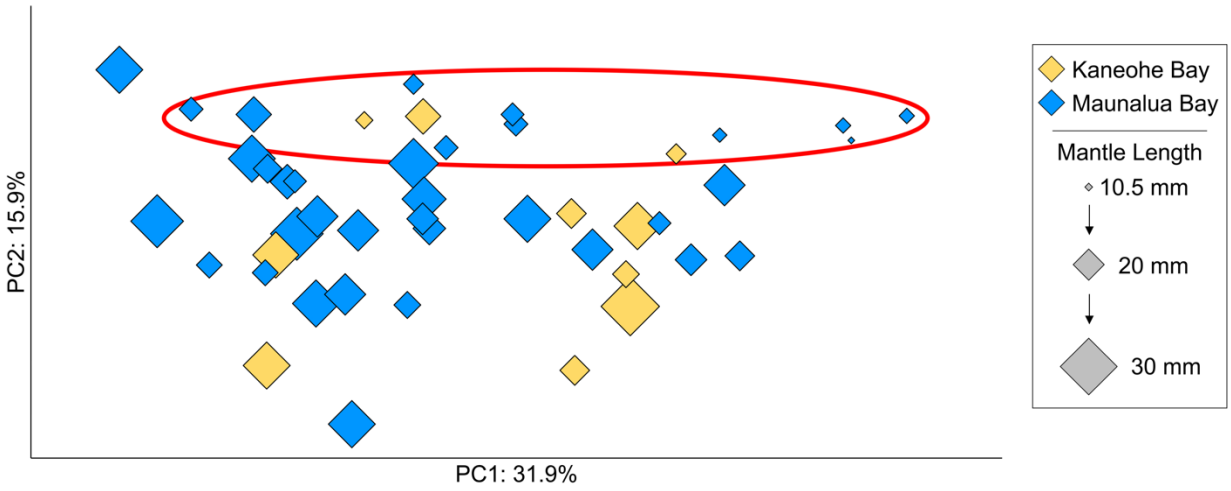


Figure 7: A Bray Curtis beta diversity analysis of ANG communities associated with various maturity levels of *E. scolopes* females finds that the smallest animal (< 18mm mantle length) ANGs cluster together but are not entirely distinct from more mature communities. Once squid approach maturity size does not appear to correlate with community composition. Mantle length is indicated by diamond size. Red oval encloses all samples of < 18 mm mantle length, as well as a couple of larger animals.

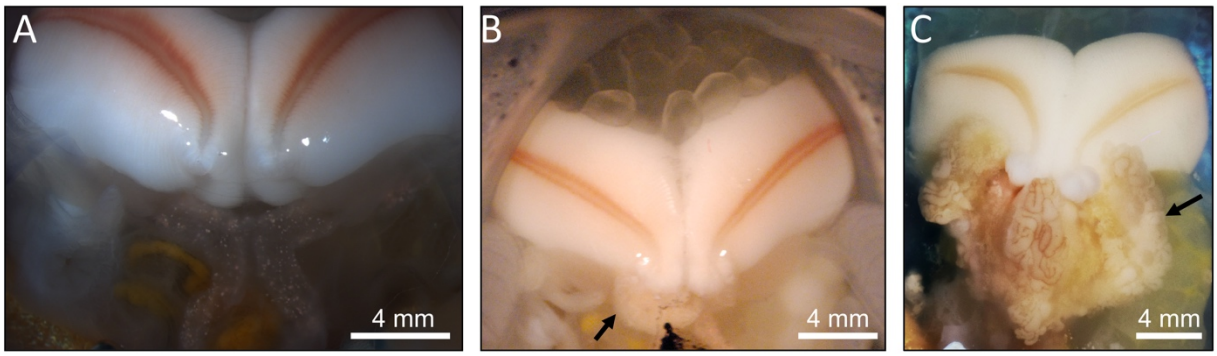


Figure 8: Mature *Euprymna scolopes* raised to adulthood under laboratory conditions at the University of Connecticut either lacked an ANG entirely (A), or developed a severely stunted and asymetrical ANG which lacked much coloration (arrow, B), in contrast to the fully formed ANG found in wild squid (arrow, C).

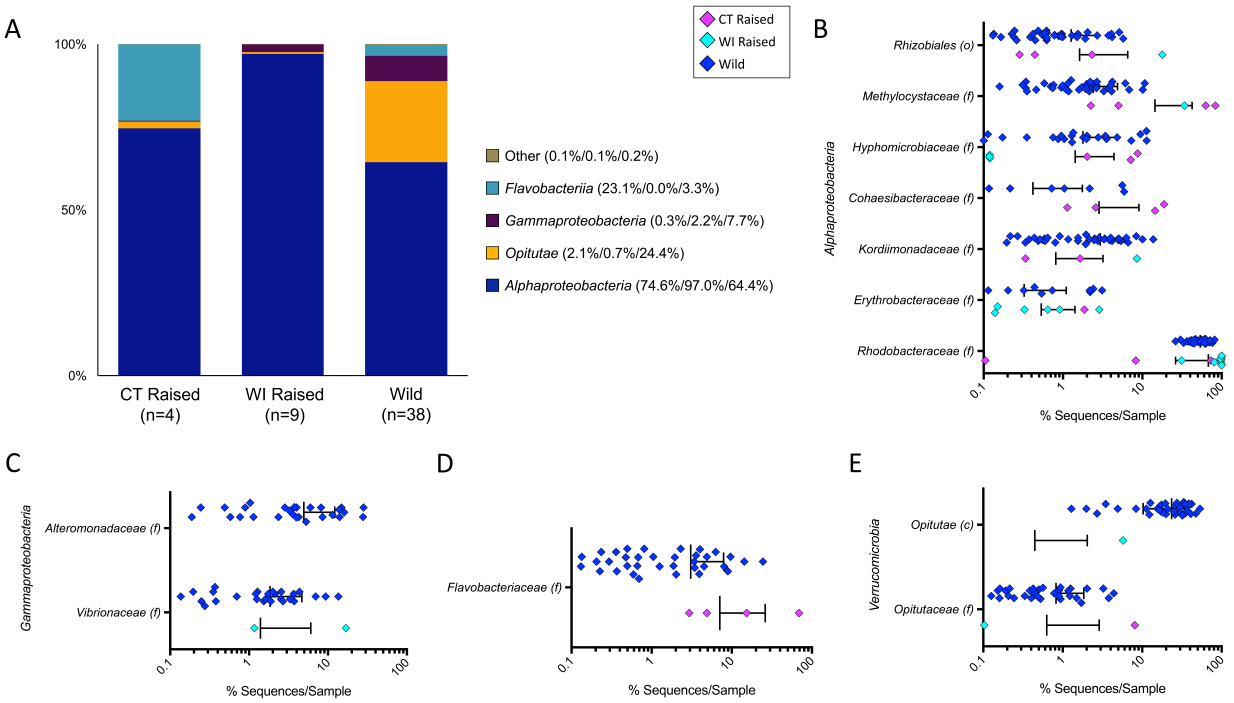


Figure 9: Taxonomic diversity of laboratory-raised *E. scolopes* ANG communities shown at the class (A) and family (B-E) levels. ANG communities of raised animals from two different labs are dominated by *Alphaproteobacteria* (A, B), but almost completely lack *Verrucomicrobia* (A, E). *Flavobacteriia* were only found in ANGs from CT-raised animals (D), while *Gammaproteobacteria* were found primarily in WI-raised animals (C). Raised animals showed high levels of variability for all taxa. Family-level plots are shown on a log scale, and include samples which contained these taxa at >0.1% abundance.

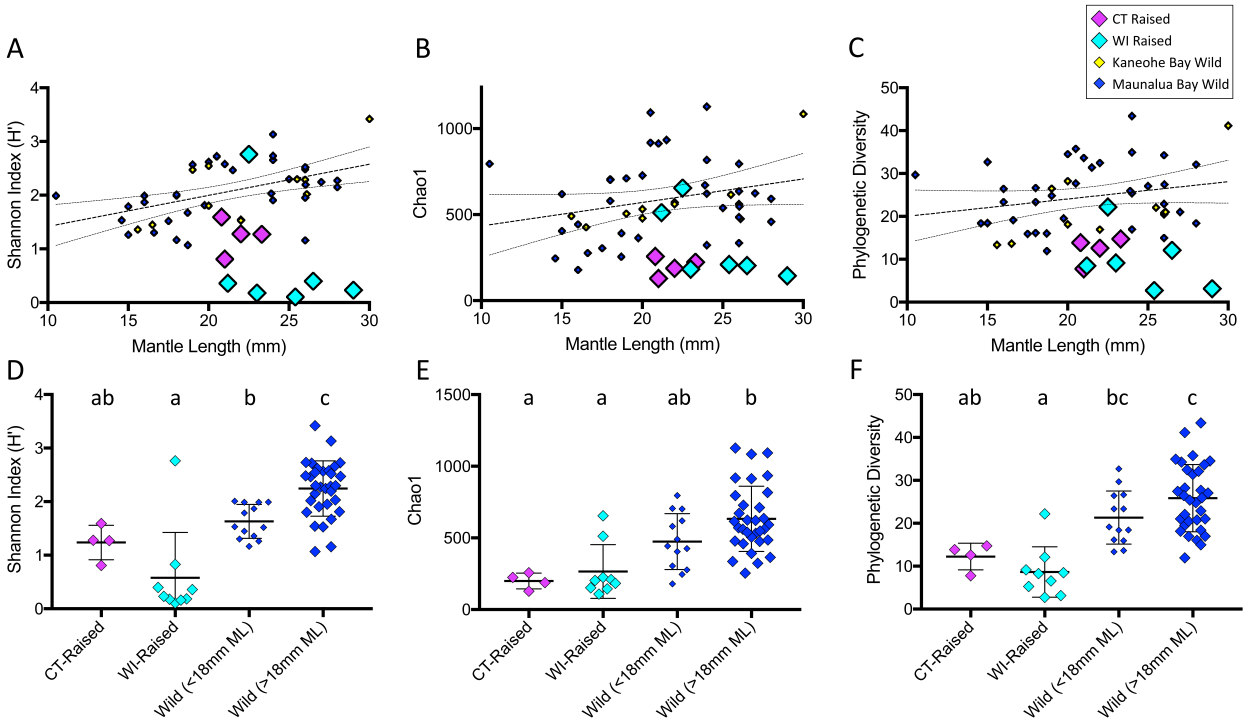


Figure 10: The ANG bacterial community of laboratory-raised animals had lower levels of alpha diversity than that of wild *E. scolopes* for all three metrics examined. The linear regression shown in A-C is based solely on wild squid communities and is provided as a reference. The CT-Raised and WI-raised ANGs had significantly lower alpha diversity than mature wild ANGs, while the WI-raised ANG alpha diversity was also significantly lower than immature wild ANGs for both Shannon Index and Phylogenetic Diversity. Letters in D-F show significantly different groups.

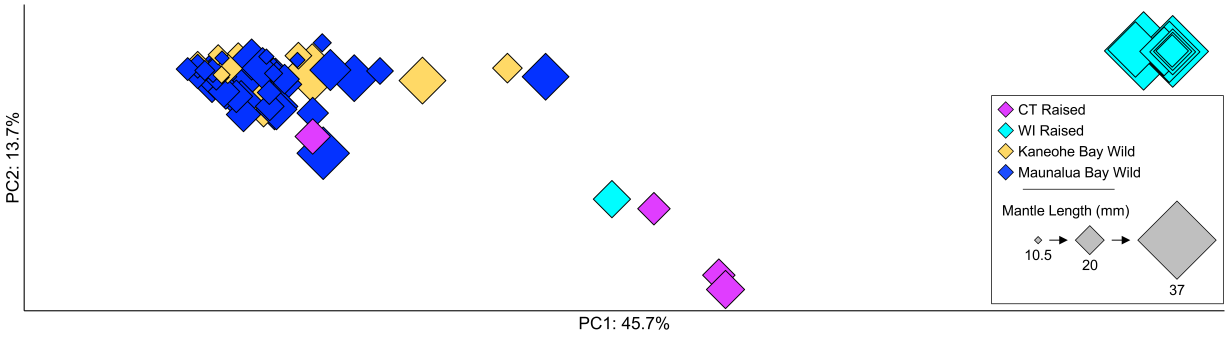


Figure 11: Bray Curtis beta diversity analysis demonstrates that the ANG bacterial composition of female *E. scolopes* raised in the lab is generally distinct from that of any maturity level of wild *E. scolopes*, regardless of the lab in which they were raised.

Chapter 5

Conclusions and Future Directions

Beneficial marine symbionts can play many different roles, providing essential nutrients to the host (i.e. *Symbiodinium* in tropical corals), camouflaging the host (i.e. *Vibrio fischeri* in the Hawaiian bobtail squid, *Euprymna scolopes,* Jones and Nishiguchi 2004), and protecting the host from infection (i.e. bacterial coating of lobster and shrimp eggs, Gil-Turnes and Fenical 1992, Gil-Turnes et al. 1989). The study of these and other symbioses has until recently relied on culture-based techniques, which has limited analyses, especially for those symbionts that have not been cultured. The recent strides in advanced sequencing technology, especially when utilized in conjunction with microscopy techniques, natural product chemistry, and ecological studies can provide a powerful tool to study all aspects of a symbiosis.

The cephalopod ANG symbiosis was first described over 100 years ago (Döring 1908), and most of the work to date has utilized culture-based techniques in conjunction with microscopy to examine the bacterial composition of the symbiosis and the development of the organ. While some research has also included clone libraries as a means of examining community composition, these studies tended to be quite limited in scope. Only in *E. scolopes* has next-generation sequencing been used to thoroughly examine community composition and to then pair that information with genomic data from cultured isolates (Collins et al. 2012, 2015). Intriguing work into a single bacterial isolate from the *E. scolopes* jelly coat has also been shown to inhibit certain marine vibrios (Gromek et al. 2016). In an effort to build on these results, I have here attempted to characterize the stability of the ANG symbiosis over space and time, to determine the function of the symbiotic community, and to examine the development of the symbiosis from the perspective of both host and bacterial partners.

Prior next generation sequencing of the *E. scolopes* ANG bacterial community relied on pooled samples (Collins et al. 2012), and was thus not able to examine individual variation, or

the stability or conservation of the community within and across populations. Here I have demonstrated that the ANG bacterial consortium is highly conserved across multiple generations of squid within a population, and that the core community of this symbiosis accounts for 79.5% of sequences recovered from the average ANG (Chapter 2). I then expanded my research to compare two *E. scolopes* populations known to experience only low levels of gene flow, and found that again the ANG symbiosis was conserved, with similar levels of alpha and beta diversity (Appendix 1). However, at a finer level, some variation in the *Gammaproteobacteria* members of the ANG was found between the two populations, with Kaneohe Bay animals containing a higher relative abundance of BD2-13, an *Alteromonadaceae* genus (Appendix 1). Some shifts were also observed in the *Alphaproteobacteria* taxa: Maunalua Bay JCs had higher relative abundances of *Leisingera* sp., while Kaneohe Bay JCs had higher relative abundances of the *Methylcystaceae* family and *Rhizobiales* order (Appendix 1). When other metrics of beta diversity were considered, the two populations were found to cluster separately by the Ochiai metric, which considers OTU presence and absence exclusively (Appendix 1). The largest shifts in the ANG composition were from non-core members, with the exception of the *Leisingera* genus and the *Rhizobiales* order.

To begin to look at the bacterial communities of a variety of cephalopods, the ANG consortia from two myopsid species, *Doryteuthis opalescens* and *Doryteuthis pealeii*, and the cuttlefish, *Sepia officinalis*, were compared to that of *E. scolopes*. The ANG community appeared most similar across host species at the class level, with all four hosts containing high levels of *Alphaproteobacteria* within the ANG (Appendix 2). However, at the family/genus level, all four communities appeared quite distinct from each other (Appendix 2). I found significantly lower levels of alpha diversity in the *S. officinalis* ANG, perhaps reflecting the fact

that these animals were laboratory-raised (Appendix 2). The two myopsid squid ANG communities were most similar to each other, despite the fact that they inhabit entirely different oceans, lending support to the hypothesis that the ANG bacterial communities will reflect host phylogeny more than geographic range (Appendix 2). However, more cephalopod species will need to be examined before firm conclusions can be drawn.

While the transfer of the ANG community to the egg jelly coat had been proposed and supported by studies that cultured similar taxa from both tissues, the best evidence to date for this transfer had been from fluorescence *in situ* hybridization of the jelly coat tissue, and was somewhat limited to investigation of only a few taxa (Collins et al. 2012). In this research, I examined the individual microbiome of the *E. scolopes* ANG community and found that the low levels of individual variation present between females was reflected in the jelly coat communities of the eggs produced by those females, demonstrating that the ANG bacterial community of a given female is deposited into the jelly coat of her eggs (Chapter 2). The jelly coat symbiotic community was stable over the course of embryogenesis, but the community also increased by an order of magnitude, demonstrating that the cells are metabolically active (Chapter 2). The stability of this community, paired with its metabolic activity, suggested that the community had an important function, and also may be allocating more energy into the production of secondary metabolites than into cell division. This trade-off hypothesis remains to be tested and will be an intriguing avenue of research in the future.

The ANG community has long been theorized to play a protective function for the eggs, preventing fouling and predation (Biggs and Epel 1991), but this hypothesis has not been tested directly. Here I demonstrate for the first time that reducing the bacterial load of eggs via antibiotic treatment leaves squid eggs vulnerable to fungal infection, while also establishing that

when such fouling occurs, the fungi can penetrate the egg capsule and jelly coat at least as far as the chorion (Chapter 3). The biofilm typically was found to take over the clutch by days 8-11 of embryogenesis and resulted in death of the embryos (Chapter 3). Closer examination revealed that fungal hyphae began to attach to the egg surface within three days of beginning antibiotic treatment (Chapter 3). The primary biofouler of the antibiotic-treated clutches was isolated and identified as the ubiquitous fungal pathogen *Fusarium keratoplasticum* haplotype FSSC-2g, and a closely related strain, haplotype FSSC-2xx, was isolated from similar experiments in Hawaiian seawater, suggesting that fusaria infections are a potential threat in the host's natural environment (Chapter 3). The biofilm bacterial community was also sequenced and determined to be distinct from the jelly coat symbiotic community (Chapter 3).

To begin to tease apart the protective effects of the jelly coat bacterial community from various host factors, I dissected the eggs into their various components, and discovered that physically removing the egg outer capsule did not result in fouling when eggs are challenged with *F. keratoplasticum* (Chapter 3). However, challenging only the naked yolk sac with *F. keratoplasticum* (removing the outer capsule and the jelly coat layers) did result in a severe biofilm which took over the eggs and killed the developing embryos, indicating that the jelly coat and its bacterial community are necessary for the prevention of fungal fouling in this system (Chapter 3). To further examine the functional potential of various members of the symbiotic consortium, I collaborated with the Balunas lab and tested organic extracts from a diverse group of ANG and jelly coat isolates. These extracts were found to differentially inhibit the growth of both *F. keratoplasticum* and the human fungal pathogen *Candida albicans* (Chapter 3). The finding that extracts from certain ANG/JC bacteria were able to inhibit *F. keratoplasticum* but

not *C. albicans*, while the reverse pattern was true for extracts from other strains, led to the development of my model for egg defense.

This model hypothesizes that different members of the ANG/JC community play different defensive roles, with some members producing secondary metabolites that inhibit *F. keratoplasticum,* while others prevent fouling by *C. albicans*, and still others against other types of fungi. Still other members of the community are able to prevent bacterial infection (Gromek et al. 2016), and host factors may also play a role as the jelly coat material can inhibit the ciliary beat of certain protists, potentially preventing protists from invading the eggs (Atkinson 1973). In the future, I hope to expand this model by testing the ability of the symbionts to prevent marine larval settlement and algal fouling, and I would also like to examine whether the eggs can actually prevent protists from fouling. Marine defensive symbioses are not uncommon, but have generally not been studied in depth. For example, while bacterial symbionts are known to protect shrimp and lobster embryos from fungal infection (Gil-Turnes and Fenical 1992, Gil-Turnes et al. 1989), very little is known about the symbionts themselves, other than the compounds that they produce. By thoroughly exploring the function of this symbiosis we demonstrate that marine symbioses can be manipulated to demonstrate function and establish that the use of such ecological manipulation can inform more observational studies.

While previous research had examined ANG development in other cephalopods using either museum specimens or laboratory-raised animals (Döring 1908, Kaufman et al. 1998), my work here is the first to examine ANG development at both a macro- and microscopic scale using wild animals, and to simultaneously examine the development of the bacterial community. Here I present my model for ANG tissue development in *E. scolopes*. My data suggest that an epithelium forms adjacent to the nidamental glands which then generates ciliated invaginations

to sample bacteria from the seawater (Chapter 4). The immature ANG appears to be poised for colonization: the tissue surface is covered with microvilli and cilia, and contains numerous ciliated invaginations, the crypts of which contain bacterial cells, and deeper in the tissue small tubules were found (Chapter 4). These ciliated invaginations appear to be temporary: larger immature ANGs lack microvilli/cilia on their surfaces and no longer contain ciliated invaginations. Future work will determine whether colonization by ANG bacteria triggers morphogenesis of the ciliated epithelium in a similar manner to what has been found for the light organ symbiosis (McFall-Ngai and Ruby 1991).

While these various host developmental changes are occurring, the bacterial community is also undergoing changes to form the mature community which is conserved in the adult population. The symbiosis shifts from a *Verrucomicrobia*-dominated community to an *Alphaproteobacteria*-dominated community as the squid matures, and community evenness also increases (Chapter 4). The ANG and bacterial community appear to be fully mature by approximately 18mm mantle length, slightly prior to full sexual maturity, which occurs around 20mm mantle length (Chapter 4).

Despite the presence of ANG bacteria in the eggs, the ANG symbiosis is hypothesized to be horizontally transmitted. Juvenile *E. scolopes* do not develop an ANG until they reach approximately 10mm mantle length (Chapter 4), approximately 1-1.5 months post-hatching (Hanlon 1997). Previous studies have found that the closest relatives to the symbiont strains were environmental (Barbieri et al. 2001), and that colonization of the ANG took an extended period of time (Kaufman et al. 1998). While my research has not proven environmental transmission, I have accumulated more evidence to support this hypothesis. I have examined the bacterial composition of the seawater and sediment in the natural environment of the squid and found that

a majority of the OTUs present in the symbiosis are also present in the environment (Chapter 2). While the ANG community of wild squid was unaffected when those squid were transferred to and maintained in the lab for up to four months (Chapter 2), squid which were raised in the laboratory had stunted or absent ANGs, and the bacterial community of those with stunted ANGs was highly variable, dependent upon the lab in which the squid were raised (Chapter 4). Raised animal ANGs also had lower alpha diversity than wild ANGs (Chapter 4). Furthermore, sequencing of the entire juvenile microbiome revealed that four out of five juvenile squid did not contain the OTU associated with the *Verrucomicrobia* contingent of the mature ANG community. Altogether I have amassed strong evidence for environmental transmission of the ANG symbiosis. While none of this evidence entirely precludes the ANG community being transferred upon hatching and stored as "symbiosis seeds" during development, future research will likely add further evidence for environmental transmission of the association.

The research presented here has for the first time demonstrated the conservation of the ANG symbiosis within and between *E. scolopes* populations, as well as throughout embryogenesis, while also establishing the link between the ANG and JC communities through the slight variations found at the individual level. I have also determined that the function of this symbiosis is defensive in nature, and have thus opened up a wide avenue for future research and natural product discovery. Finally, my examination of the development of the ANG and its symbiotic community have provided insight into ways that this symbiosis might be manipulated, while also accumulating evidence for the horizontal transmission of the symbiotic community. The ANG symbiosis provides a tractable model for the development, maintenance, and functional capacity of a complex marine symbiosis. My research thoroughly demonstrates that integrating knowledge of community composition, functional ecology and natural product

discovery can help us to understand the roles of various players within a symbiosis. Collaborative science can lead to insights that would not be possible working alone within a single field, and the research presented here will hopefully encourage others to approach their work from a new perspective.

Appendix I

Reproductive system symbiotic bacteria are conserved between two populations of *Euprymna scolopes* from Oahu, Hawaii

Abstract

Female Hawaiian bobtail squid, *Euprymna scolopes*, harbor a symbiotic bacterial community in the accessory nidamental gland (ANG), a reproductive system organ. This community is known to be stable over several generations of wild-caught bobtail squid but has to date only been examined for one wild population in Maunalua Bay, Oahu, Hawaii. This study examined the ANG bacterial community for a second population of *E. scolopes* from Kaneohe Bay, Oahu, Hawaii which is geographically and genetically isolated from the population previously examined. The bacterial communities from both populations were found to be similar in richness, evenness, phylogenetic diversity, and overall community composition. However, an *Alteromonadaceae* genus, BD2-13, was significantly higher in relative abundance in the Kaneohe Bay population than in the Maunalua Bay population, and a few *Alphaproteobacteria* taxa also shifted in abundance between the populations. This variation could be due to local adaptation to differing environmental threats, to localized variability, or to functional redundancy between the taxa present. The overall stability of the community between the populations further supports the crucial functional role that has been demonstrated for this symbiosis, while the few variable taxa open up potential avenues for additional drug discovery candidates.

Introduction

Euprymna scolopes, the Hawaiian bobtail squid, is endemic to the Hawaiian archipelago. These bobtail squid depend on their well-studied light organ symbiont, *Vibrio fischeri*, to avoid predation (Jones and Nishiguchi 2004), and their reproductive symbiotic community to prevent fouling and infection of their eggs throughout embryogenesis (Chapter 3). The reproductive bacterial symbionts are found in adult females in the accessory nidamental gland (ANG). This organ deposits bacteria into the jelly coat (JC) of eggs (Kerwin and Nyholm 2017) just before

eggs are deposited onto the substrate. The JC bacteria inhibit fungal fouling and certain marine bacteria via the production of a variety of secondary metabolites (Chapter 3, Gromek et al. 2016).

Research into these bobtail squid and their symbionts depends on two populations of *E. scolopes* from the island of Oahu, Hawaii (Figure 1). Maunalua Bay is the current primary collection source for *E. scolopes*, but animals are also frequently collected from Kaneohe Bay. Maunalua Bay (MB), located on the southern coast of Oahu, is a shallow sand flat reaching approximately 600 meters from shore to the reef crest. Kaneohe Bay (KB) is located to the north of MB on the eastern coast of Oahu, and is the only true barrier reef in the Hawaiian archipelago (Kay and Palumbi 1987). While bobtail squid are found in several discrete spots throughout KB (Lee and Ruby 1994), they are generally collected from a smaller sand flat reaching approximately 120 meters from shore to a deeper channel.

These two host populations are not exceedingly distant from each other geographically (Figure 1). However, the two populations exhibit low levels of gene flow and several morphological differences have been demonstrated (Kimbell et al. 2002). MB females, eggs, and juveniles are significantly larger than those from KB (Kimbell et al. 2002). These populations are also known to contain different strains of *V. fischeri* in their light organs (Wollenberg and Ruby 2004). The low levels of gene flow, the host and light organ symbiont differences between these two sites, and the ease of collection from both sites, all make these populations a good source for examining variation in the ANG symbiotic community.

In this study, we collected squid from KB. As maintaining squid in the laboratory is not known to affect the ANG consortium (Kerwin and Nyholm 2017), squid were either sacrificed in Hawaii or transported to Connecticut and maintained for up to four months. Eggs were collected

from laboratory-maintained animals. The ANG and egg jelly coat (JC) communities were compared to previously published samples from MB animals.

Methods

Ten sexually mature female squid (ranging in mantle length from 19mm to 30mm) were collected from Kaneohe Bay (KB, 21°16'51.42" N; 57°43'33.07" W) using dip nets and were immediately transferred to Kewalo Marine Laboratory. Squid were either sacrificed within two days or were shipped to Connecticut and maintained in our squid facility for up to four months. Lab-maintained females were regularly mated and kept in individual tanks to allow clutches to be matched to females. Bobtail squid were anesthetized in two percent ethanol prior to sacrifice. Egg clutches were collected and dissected within 12 hours of deposition. All tissues were surface sterilized in 99% ethanol and filter-sterilized squid Ringer's solution (FSSR, Collins et al. 2012) to remove transient bacterial contaminants.

DNA extraction from ANGs (n=10) and egg JCs (n=20) was completed as previously described (Kerwin and Nyholm 2017). Briefly, ANGs were homogenized in FSSR and then underwent differential centrifugation to separate the bacterial cells from host tissue. DNA extraction of the bacterial component was completed using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) with bead-beating (Mini-Beadbeater-16, BioSpec Products, Bartlesville, OK). Ten JCs were separated from their outer egg capsules and yolk sacs and pooled in a bead-beating tube. The JCs were flash-frozen to -80°C for a minimum of 30 minutes and DNA was extracted using the MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI) with bead-beating and an increased concentration of Proteinase K (0.833 µg/ml).

Extracted DNA was amplified using barcoded primers developed for the V4 region of the 16S rRNA gene by Caporaso et al. (2012), and sequenced on an Illumina MiSeq (Illumina, San

Diego, CA) following established protocols (Benjamino et al. 2016, Nelson et al. 2014). The UConn Microbial Analysis, Resources and Services facility (MARS) processed some of the samples. Extraction and PCR controls were processed and sequenced simultaneously with all samples as described in Chapter 4.

Sequencing data was analyzed using QIIME (Caporaso et al. 2010). Greengenes (2013-08 release, DeSantis et al. 2006) and de novo methods were used to assign operational taxonomic units (OTUs) at the 97% identity level (Benjamino and Graf 2016). Samples were rarified to 10,000 sequences. Alpha diversity was analyzed in QIIME, and the log2 Shannon Index was converted to a natural log Shannon Index. Alpha diversity plots were created and differences in alpha diversity were tested using two-way ANOVA with post-hoc Tukey tests in PRISM. Beta diversity was analyzed using the Bray-Curtis metric, with community composition similarity tested by ANOSIM in QIIME and NMDS plots created in R using the VEGAN package (Oksanen et al. 2016). Beta diversity was also analysed using the Ochiai metric and weighted and unweighted UNIFRAC metrics in QIIME, with community composition similarity tested by ANOSIM. KB sequences were compared to MB sequences previously published and available under project ID PRJEB14655 (Kerwin and Nyholm 2017).

Results

Kaneohe Bay bobtail squid had a similar ANG and JC community composition to those found in Maunalua Bay. ANG and JC samples clustered together on an NMDS plot based on the Bray Curtis metric (Figure 2a), and an ANOSIM analysis did not indicate any dissimilarity between the populations (R=0.062, p=0.07). In agreement with a previous study, the ANG and JC bacterial communities within the KB population are similar to each other, although in this case a small shift in the community composition was noted (Figure 2b, R=0.209, p=0.02, Kerwin

and Nyholm 2017). A closer examination of the taxa found in the Kaneohe Bay ANG and JC communities found that the *Methylocystaceae* (family of *Alphaproteobacteria*) and *Opitutae* (class of *Verrucomicrobia*) were significantly higher in the ANG than in the JC (1.8% vs 0.5%, t_{30}=4.177, p=0.002, and 24.8% vs 9.6%, t_{30}=3.811, p=0.003, respectively), while the *Flavobacteriaceae* are significantly lower in the ANG than in the JC (1.1% vs 3.5%, t_{30}=4.132, p=0.002), and none of these differences appear to be the result of outlier samples. The clustering of ANG and JC communities by their associated female was less clear in the Kaneohe Bay population than has been shown previously for the Maunalua Bay population (Kerwin and Nyholm 2017). While the Bray Curtis analysis of ANG and JC groups did not indicate well-defined groupings (Figure 3b), an ANOSIM analysis demonstrated that at least some of the female groups were distinct from each other (R=0.812, p=0.001). In the previously published Maunalua JC dataset, we observed radiation from the early stage JCs out to the later stage JCs within female groups. Therefore, the lack of clarity in the NMDS female groupings in this Kaneohe Bay dataset could be due to this dataset only including JCs from the earliest stage of embryogenesis (Day 0). To confirm this the MB dataset was reanalyzed only including Day 0 JCs and their ANG pair, and as predicted the female groupings were much less distinct (Figure 3a).

Alpha diversity was also similar between KB and MB populations, both for ANG and JC bacterial communities (Figure 4). Three types of alpha diversity were analyzed to give a broad portrait of within sample diversity. Bacterial richness and evenness (H'), phylogenetic diversity (PD) and richness informed by the number of rare OTUs (Chao1) were all similar between the two populations and two tissue types, when analyzed via two-way ANOVA (Figure 4). The

larger spread in MB JC samples compared to other sample types is again attributed to including a wider set of JCs from different stages of embryogenesis.

While both alpha and beta diversity metrics found no differences between the Kaneohe and Maunalua Bay ANG and JC bacterial communities, some differences in the relative abundances of certain taxa were observed. The KB and MB ANGs are both dominated by *Alphaproteobacteria* (61.6% vs 65.1%) and *Verrucomicrobia* (21.8% vs 24.9%, Figure 5a). The JCs from both populations have higher levels of *Alphaproteobacteria* (74.83% KB vs 70.9% MB) and lower levels of *Verrucomicrobia* (10.5% KB vs 7.9% MB) than the ANGs (Figure 5a). However, *Gammaproteobacteria* in KB ANGs account for a significantly higher proportion of the community than in MB ANGs (15.7% vs 4.6%, t_{34}=4.635, p=0.0002, Figure 5b). The higher proportion of *Gammaproteobacteria* in KB was due to an *Alteromonadaceae* genus (BD2-13, 13.1% KB vs 2.0% MB, t_{34}=5.023, p=0.0003, Figure 6a). A similar difference was seen in the JC for the same genus (9.4% KB vs 2.0% MB, t_{53}=5.588, p=0.00001, Figure 6b).

Surprisingly, two *Alphaproteobacteria* taxa were significantly higher in the Maunalua Bay JC than in the Kaneohe Bay JC (*Methylocystaceae* (f) 1.8% MB vs 0.5% MB, t_{53}=3.639, p=0.01; *Rhizobiales* (o) 0.6% MB vs 0.3% KB, t_{53}=3.107, p=0.04), while a third (*Leisingera* sp.) was significantly higher in the Kaneohe Bay JC than in the Maunalua Bay JC (0.01% MB vs 0.8%, t_{53}=3.085, p=0.04, Figure 6b). No similar results were found for the ANG community. These changes within the *Alphaproteobacteria* taxa indicate that the *Alphaproteobacteria* community may also shift slightly between the populations, although none of these taxa account for substantial proportions of the communities. The *Leisingera* sp. difference appears to be due in large part to a few outliers within the Kaneohe Bay JCs.

Additional beta diversity metrics were used to analyze the dataset given the differences discussed above. Principle coordinate analysis using the Ochiai metric found significant differences between the two populations (Figure 7). The Ochiai analysis is based on OTU presence/absence. The majority of OTUs absent in either MB or KB samples, were found in only a very small proportion of the other sample type. Of the six OTUs absent in MB samples which were present in at least 33% of KB samples, three belonged to the BD2-13 genus, one was an unclassified *Gammaproteobacteria*, and the last two were *Kordiimonadales*. Only four OTUs were present in at least 33% of MB samples, but missing from all KB samples, and of those, two were *Opitutae*, and two *Rhodobacteraceae*.

Discussion

The KB bobtail population is genetically isolated from the MB host population, potentially giving the ANG bacterial community room to adapt and reflect different environmental constraints. In this study, we find that such adaptation has largely not occurred in the ANG community. The ANG and JC communities of KB and MB animals are dominated by *Alphaproteobateria* from the *Rhodobacteraceae* family and *Verrucomicrobia* from the *Opitutae* class (Figure 5). Alpha and most beta diversity metrics showed no differences between the populations, demonstrating that the communities are similar in terms of species richness, evenness, phylogenetic diversity and community composition based on OTU abundance (Figures 2 and 4). However, the Kaneohe Bay population contained a significantly higher proportion of *Gammaproteobacteria* from the BD2-13 genus (a member of the *Alteromonadaceae*, Figure 6). Altogether this data suggests that the community is stable between different populations of *E. scolopes*, with the slight differences in community composition potentially reflecting local

adaptation to differing environmental conditions, localized variability, or functional redundancy between the taxa present.

The finding that the biggest variation between the two populations was due to an increase in proportion of *Alteromonadaceae* was especially interesting, as *Alteromonadaceae* are known to produce many secondary metabolites. The production of secondary metabolites by members of this symbiosis has been shown to protect the *E. scolopes* eggs from fungal fouling (Chapter 3). While *Actinobacteria* and to a lesser degree *Cyanobacteria* have been responsible for the majority of natural product drug discovery in marine ecosystems, the *Alteromonadaceae* are responsible for a high proportion of *Proteobacteria* natural products, especially when compared to the *Alphaproteobacteria* (Jensen and Fenical 1994, Williams 2009, Desriac et al. 2013). The higher prevalence of *Alteromonadaceae* in the KB ANG defensive symbiosis is intriguing and could provide an increased source for natural product discovery in the future. The potential for functional redundancy between these alteromonads and some other bacterial groups which take the place of these bacteria in the MB ANG could also provide insight into other bacterial species that could bear further investigation in the future.

In many other systems, symbionts have been found to diverge between different populations to various degrees. Gut communities frequently vary due to differences in diet between populations, as is seen in the human gut microbiome (Yatsunenko et al. 2012, Lozupone et al. 2012), or in the juvenile Atlantic salmon, *Salmon salar*, where the gut microbiome varied only in *Mycoplasmataceae* OTUs (Llewellyn et al. 2016). The hindgut microbiota of termites, *Reticulitermes flavipes*, from different but nearby populations, showed similar abundance patterns for the core taxa but did exhibit variation hypothesized to allow the termites to distinguish nest mates from invaders (Benjamino and Graf 2016). Obligate nutritional

endosymbionts such as *Candidatus* Erwinia dacicola in the olive fruit fly, *Bactrocera oleae,* are present at the same levels in all wild populations examined (Estes et al. 2012). Other obligate endosymbionts, such as *Symbiodinium* in corals, can vary at the strain level (Frade et al. 2008).

In a symbiosis functionally similar to that of the ANG, the epithelial bacterial community of *Hydra oligactis* has been shown to provide protection from fungal fouling (Fraune et al. 2015). The *H. oligactis* and the ANG symbiosis appear to share similar population dynamics. A comparison of *Hydra oligactis* from two German lakes found that the populations contained many of the same bacterial taxa, and grouped together apart from the community of *Hydra vulgaris* from one of the same lakes (Fraune and Bosch 2007). However, each population did contain some bacterial taxa not found in the other population (Fraune and Bosch 2007), similar to what we have shown here for the two population of *E. scolopes*. In the pea aphid, *Acyrthosiphon pisum,* secondary symbionts play defensive roles against fungal pathogens, but which symbiont taxa are present varies widely by population (Zytynska and Weisser 2016).

The selective pressure exerted on a defensive symbiosis will largely depend on how prevalent the threat is in the natural environment of the host, and on the fitness effect posed by the threat. If fungal fouling only rarely impacts *E. scolopes* clutches or if that fouling does not negatively impact host survival or fitness, then the selective pressure to conserve the symbiosis throughout the species will be low. Varying environmental conditions between populations could result in varying levels of selective pressure. The largely conserved ANG symbiosis between these two populations of *E. scolopes* may reflect the strong threat of egg fouling by fungi and/or other microbes. In the future, *in situ* experiments investigating fouling levels for antibiotic-treated clutches returned to MB and KB could lend insight into the occurrence of fouling. Examination of bobtail populations from other islands in the Hawaiian archipelago could also

enhance our understanding of the stability of the ANG community across the species. The strong conservation of the *E. scolopes* ANG bacterial consortium across populations provides further evidence to suggest an obligate nature of this defensive symbiosis under natural environmental conditions. The overall stability of the community between the populations further supports the crucial functional role that has been demonstrated for this symbiosis, while the few variable taxa open up potential avenues for additional drug discovery candidates.

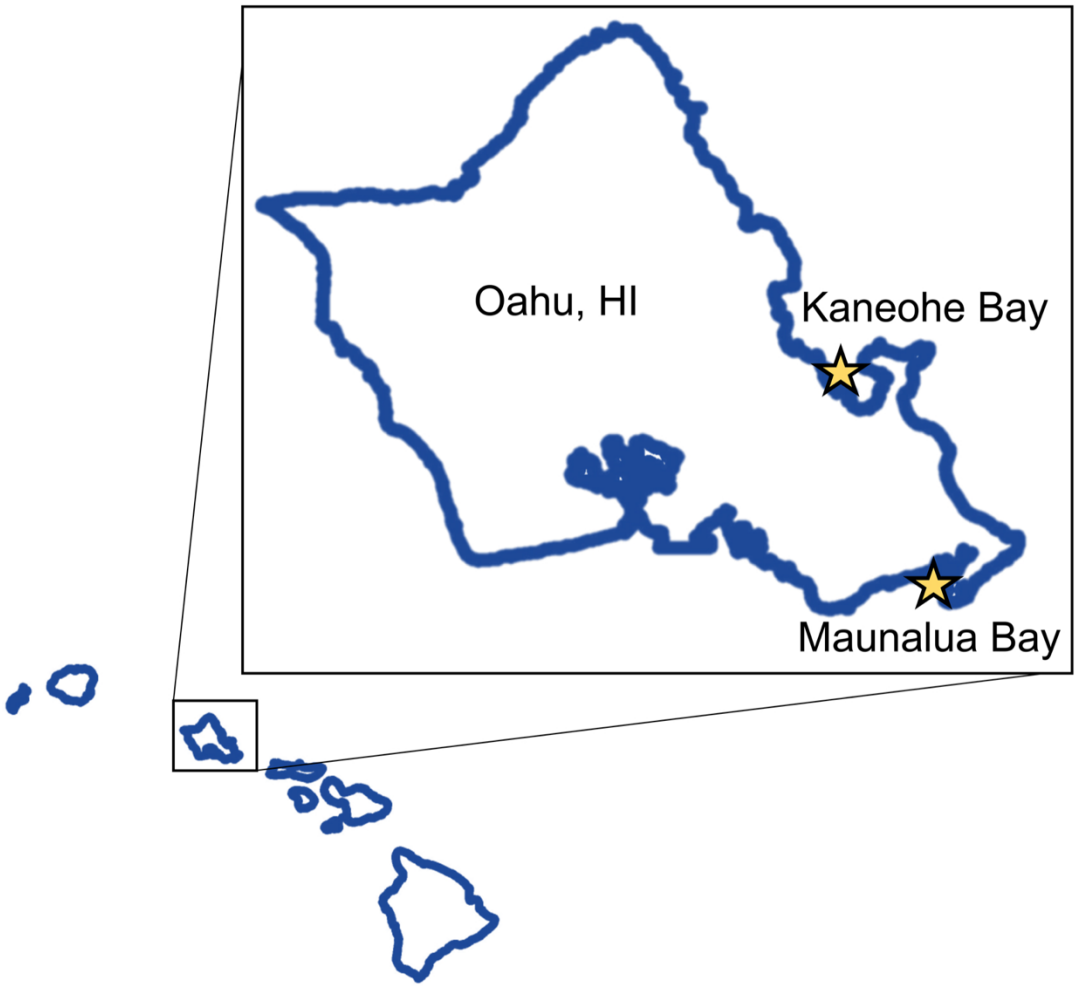


Figure 1. Map of Oahu, Hawaii, showing locations of two squid populations, Kaneohe Bay (21°16’51.42” N; 157°43’33.07” W) and Maunalua Bay (21°26’3.36”N, 157°47’20.78”W).

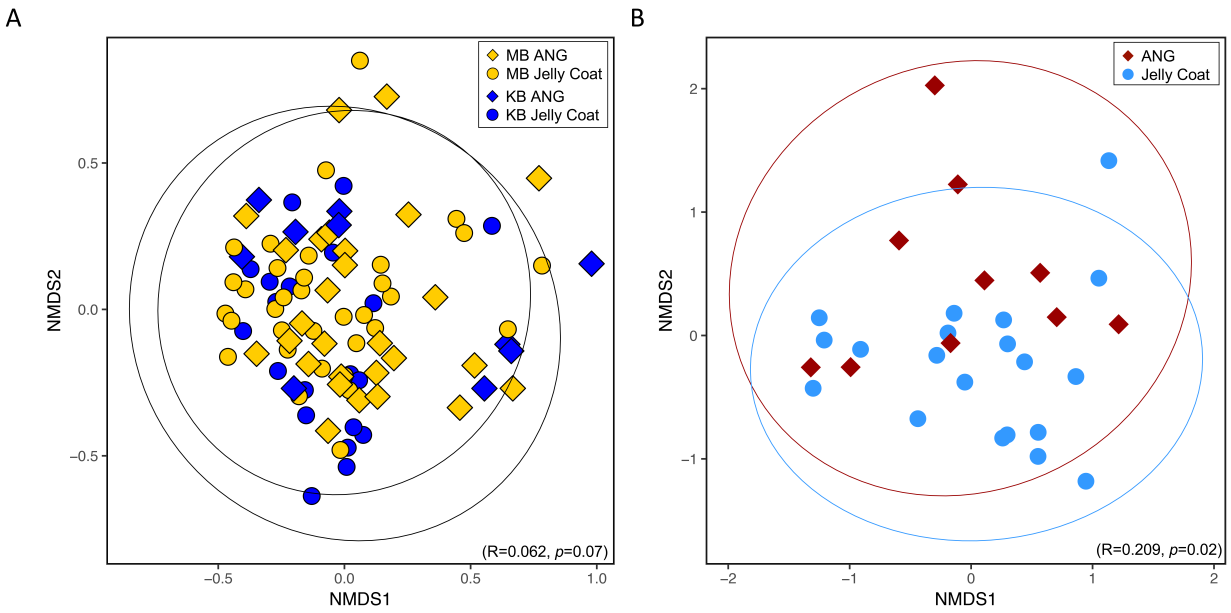


Figure 2. Bray Curtis beta diversity analysis of Kaneohe Bay ANGs and JCs. The overall community composition of Kaneohe Bay and Maunalua Bay ANGs and JCs are not distinct (A). ANG and JC bacterial community composition overlap substantially in Kaneohe Bay (B). Ellipses represent 95% confidence intervals.

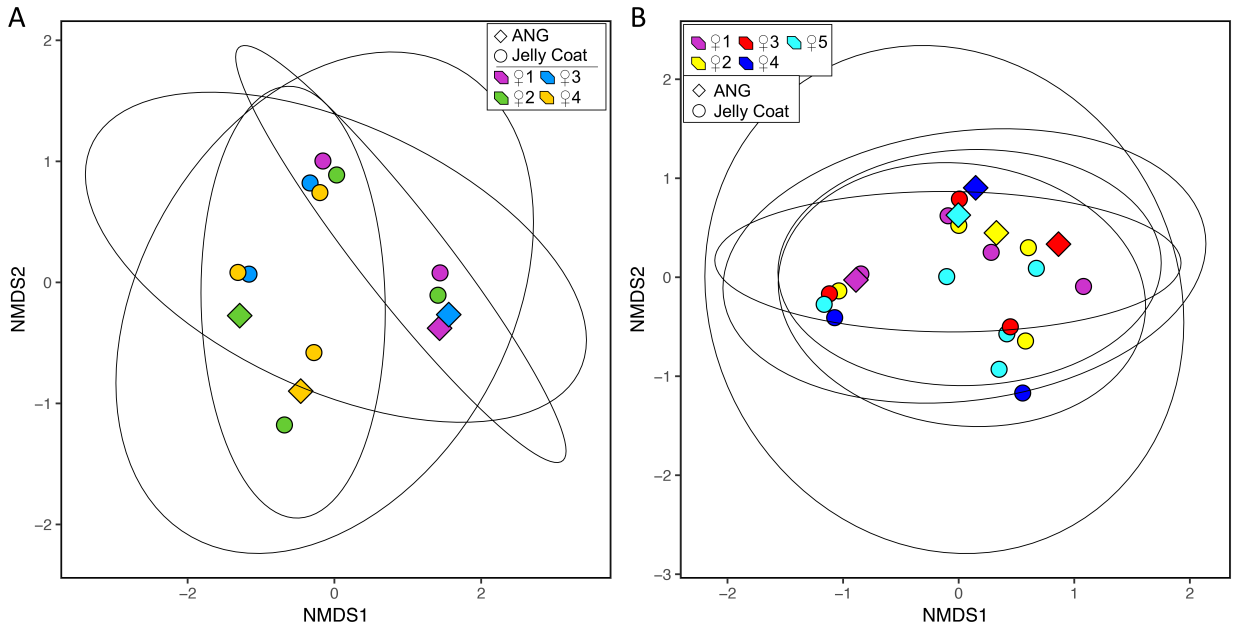
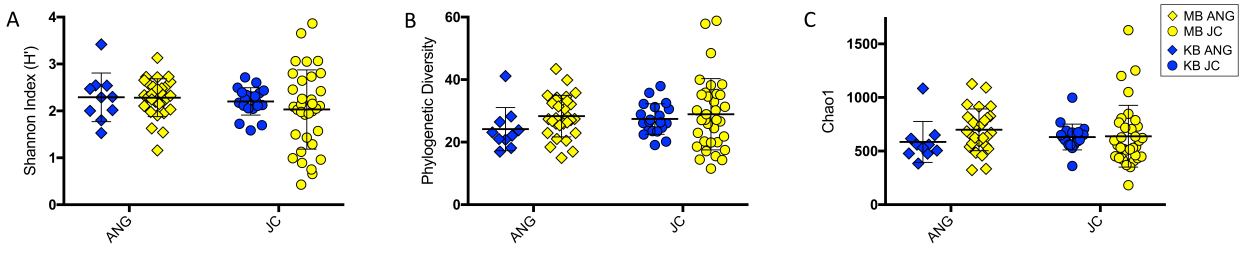


Figure 3. Beta diversity analysis of Maunalua Bay (A) and Kaneohe Bay (B) ANG and JC groups by associated female. Bacterial communities do not cluster by female in Kaneohe Bay, and only loosely cluster by female in Maunalua Bay. Ellipses represent 95% confidence intervals. Original analysis of Maunalua Bay ANG/JC groups included JCs from all stages of embryogenesis, while this analysis only includes JCs from Day 0.



Source	df	MS	F-ratio	p-value
Population	1	0.15	0.43	0.51
Tissue Type	1	0.54	1.48	0.23
Pop. x Tissue Type	1	0.12	0.34	0.56
Residual	89	0.36		

Source	df	MS	F-ratio	p-value
Population	1	151.7	2.11	0.15
Tissue Type	1	68.15	0.95	0.33
Pop. x Tissue Type	1	32.68	0.45	0.50
Residual	89	72.01		

Source	df	MS	F-ratio	p-value
Population	1	69060	1.40	0.24
Tissue Type	1	928.3	0.02	0.89
Pop. x Tissue Type	1	53375	1.08	0.30
Residual	86	49431		

Figure 4. Alpha diversity analysis of Kaneohe Bay ANGs and JCs. Two-way ANOVA found no effect of population or tissue type on bacterial community richness/evenness (A), phylogenetic diversity (B), or on richness calculated from the number of rare taxa present (C). Thick bars indicate mean, thin bars standard deviation.

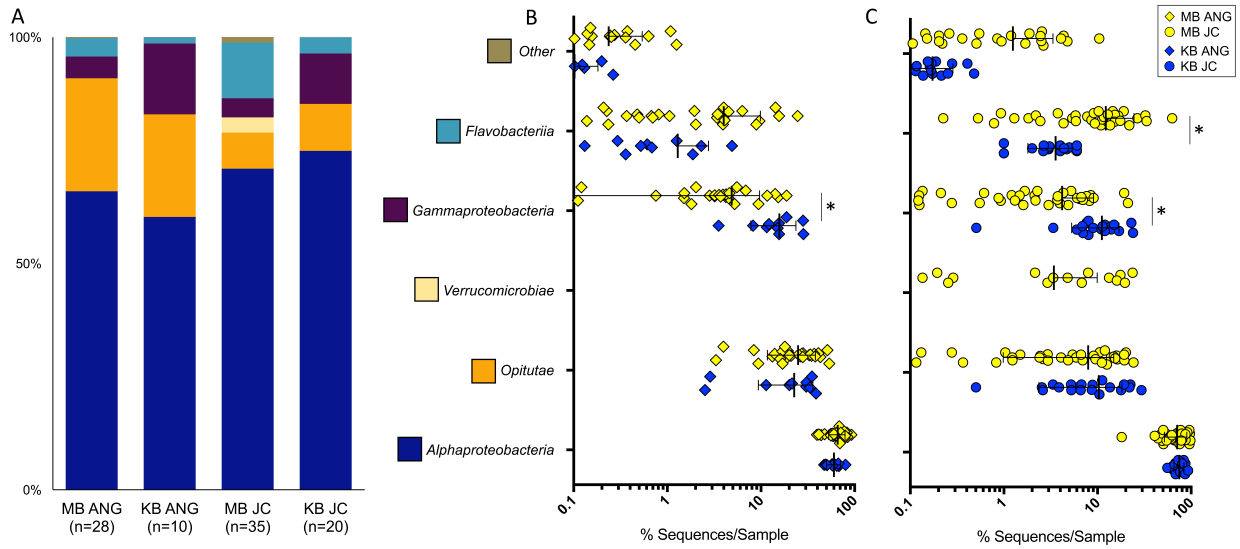


Figure 5. Kaneohe Bay (KB) ANG and JC bacterial communities resemble those seen in Maunalua Bay (MB, A). KB animals have significantly more *Gammaproteobacteria* in their ANGs than do MB animals (t_{36}=5.129, p<0.0001, B). KB animals also have significantly more *Gammaproteobacteria* in their JCs than do MB JCs (t_{53}=4.73, p=0.0001), and also have less *Flavobacteriia* in their JCs (t_{53}=3.138, p=0.01, C). Taxa presented at class level, scatter plot presented on a log scale to show variation for taxa present at lower average abundances. Thick bars represent mean, thin bars standard deviation (B, C).

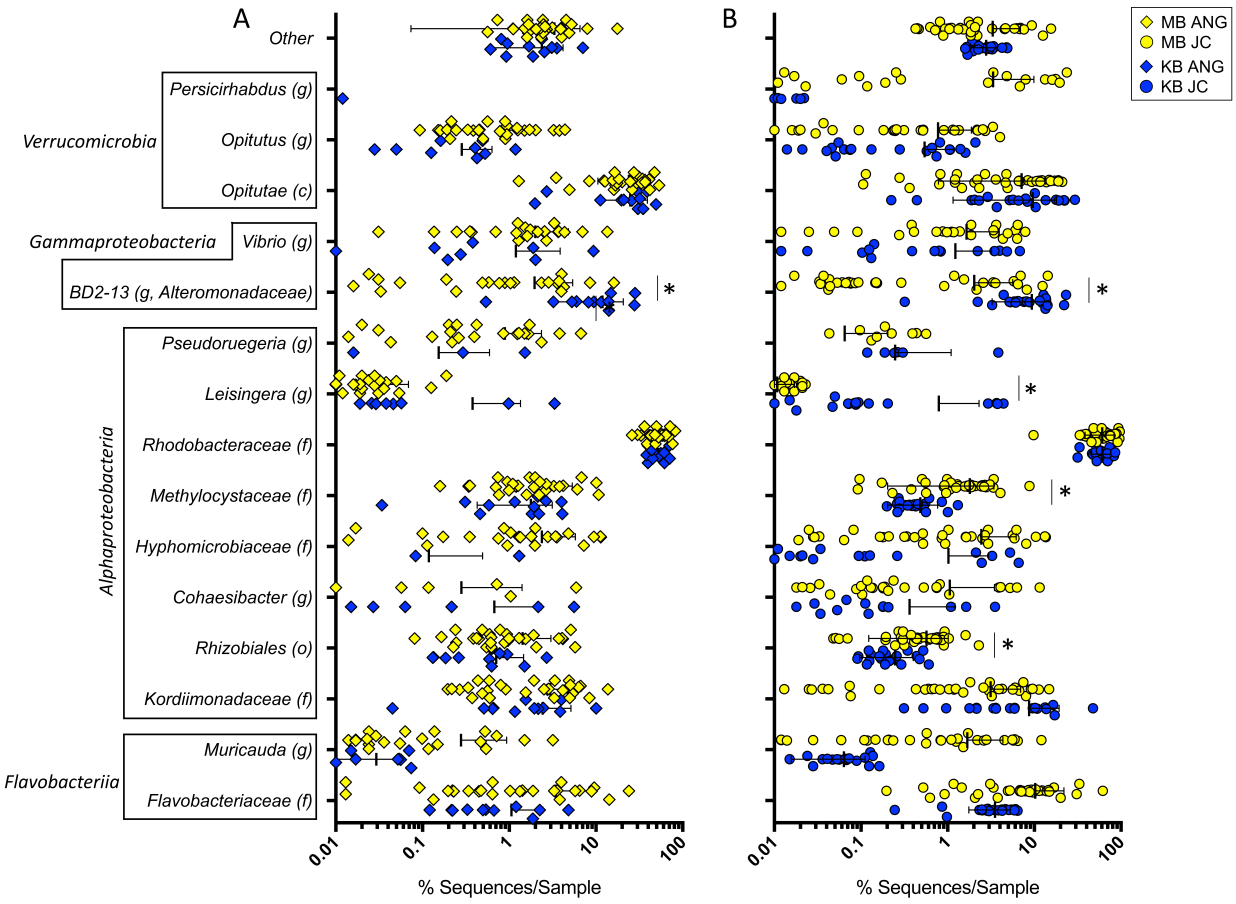


Figure 6. The higher abundance of *Gammaproteobacteria* in the Kaneohe Bay (KB) ANG and JC bacterial communities is due to a shift in BD2-13, a genus from the *Alteromonadaceae* family. BD2-13 (t_{38}=5.22, p=0.0001) is significantly more abundant in KB ANGs (A), and in KB JCs (t_{53}=5.612, p=0.00001, B). One *Alphaproteobacteria* taxa (*Leisingera:* t_{53}=3.085, p=0.04) was also significantly higher in KB JCs (B), while two others were significantly lower in KB JCs (*Rhizobiales:* t_{53}=3.107, p=0.04, and *Methylocystaceae*: t_{53}=3.639, p=0.009). Taxa presented at finest level obtained (c-class, o-order, f-family, g-genus), scatter plot presented on a log scale to show variation for taxa present at lower average abundances. Thick bars represent mean, thin bars standard deviation.

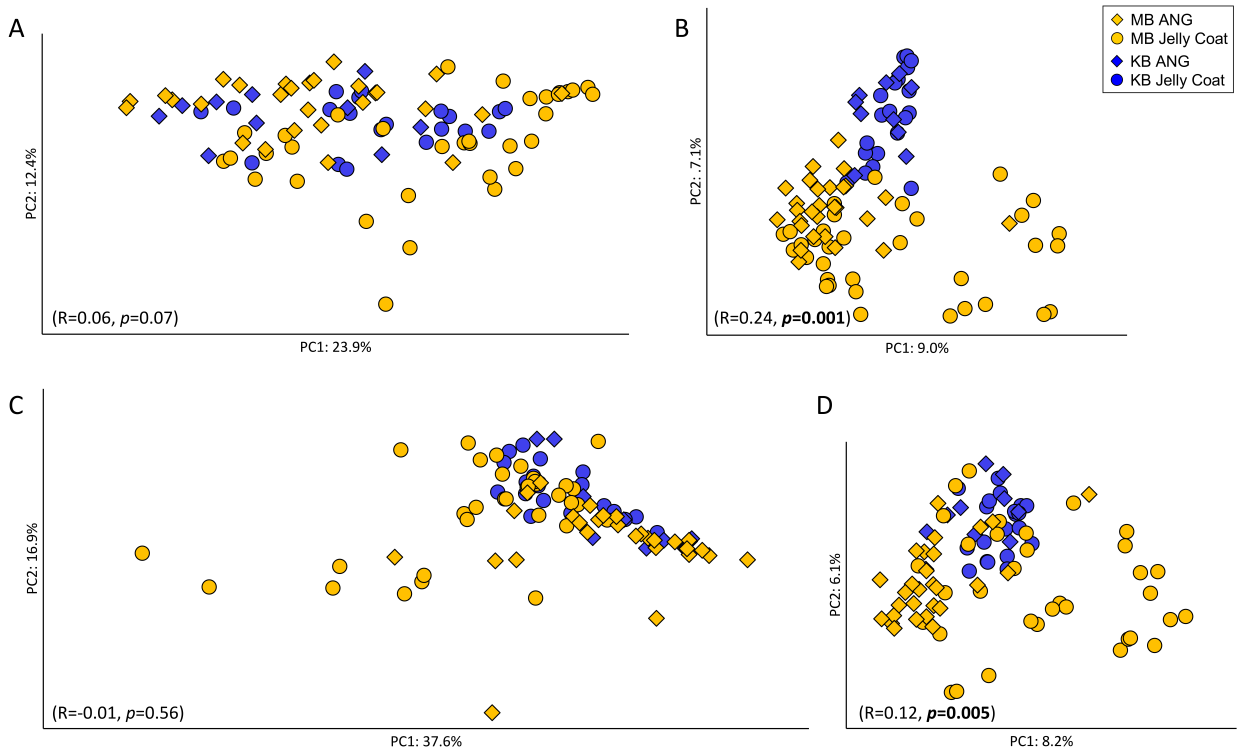


Figure 7. Principle coordinate analysis of various beta diversity metrics comparing Kaneohe Bay and Maunalua Bay ANGs and JCs. Bray Curtis (A, OTU abundance) and weighted UNIFRAC (C, phylogeny/OTU abundance), and unweighted UNIFRAC (D, phylogeny) metrics show no distinction between the populations. However, in Ochiai (B, OTU presence/absence) analysis the two populations did cluster separately.

Appendix II

ANG bacterial community variation between cephalopod species

Abstract

The accessory nidamental gland (ANG) is a symbiotic reproductive system organ found in many species of squid, bobtail squid, and cuttlefish. To date the community has only been investigated in depth for the Hawaiian bobtail squid, *Euprymna scolopes*. In this study, the V4 region of the 16S rRNA gene was sequenced in order to characterize the ANG bacterial communities of three other cephalopod species, *Sepia officinalis*, *Doryteuthis opalescens*, and *Doryteuthis pealeii*. The bacterial composition of these ANG communities was found to be distinct from each other and from that of *E. scolopes*, and to share few operational taxonomic units (OTUs) across species lines. However, similar levels of alpha diversity were observed for all but *S. officinalis*, and some taxonomic similarity within the communities, such as the high relative abundance of *Alphaproteobacteria* and more specifically the *Rhodobacteraceae*, was demonstrated for all four host species. The ANG bacterial community does not appear to be strongly conserved across cephalopod lineages, but further sampling efforts are necessary to strengthen this conclusion.

Introduction

Symbioses frequently cross species lines and play similar functions in taxonomically-related hosts. For example, the obligate nutritional symbiont, *Buchnera aphidicola*, is found in almost all species of aphids (Munson et al. 1991). Similarly, tropical reef-building corals have an obligate symbiosis with a dinoflagellate from the *Symbiodinium* genus, although the exact species/strain(s) may vary between coral species. However, the presence of so called secondary symbionts, which may increase fitness under certain environmental conditions, are generally not as conserved across species lines. In aphids, secondary symbionts play various defensive roles, such as protection from fungal infection or resistance to parasitoid wasps, as well as increasing

recovery from heat shock (Zytynska and Weisser 2016). As these roles are context-dependent, the presence of these symbionts varies widely between host species (Zytynska and Weisser 2016). Complex bacterial symbiotic communities seem to be less stable over host species, possibly due to functional redundancy. In both coral and sponge hosts, only a few bacterial species appear to be conserved across many host species, despite the high richness of both these communities (Ainsworth et al. 2015, Thomas et al. 2016). Identifying conserved members of a community may grant insight into which bacteria are functionally obligate symbionts, and also reveal bacteria that play similar roles in different hosts.

The accessory nidamental gland (ANG) is a female reproductive organ found in seven different cephalopod families (Lindgren et al. 2012). This organ contains a diverse bacterial community that is conserved across populations of the Hawaiian bobtail squid, *Euprymna scolopes* (Appendix I). This bacterial community is deposited into the jelly coat of the bobtail squid eggs (Kerwin and Nyholm 2017) and plays an important defensive role, protecting the developing embryos from fungal fouling (Chapter 3). The ANG community composition has been investigated in depth using next generation sequencing only in *E. scolopes*, where it is known to consist primarily of *Alphaproteobacteria* and *Verrucomicrobia*, with *Gammaproteobacteria* and *Flavobacteriia* being more minor members (Collins et al. 2012, Kerwin and Nyholm 2017).

In other cephalopods, the community has been investigated using fluorescence *in situ* hybridization (FISH), 16S clone libraries, and culture-based methods. These methods have demonstrated that the loliginid ANG community is made up of *Alphaproteobacteria* and *Gammaproteobacteria* (Barbieri et al. 2001, Barbieri et al. 1996, Lum-Kong and Hastings 1992, Pichon et al. 2005), while in *Sepia officinalis* the ANG community is composed of Gram positive

bacteria and *Alphaproteobacteria* (Grigioni et al. 2000). While these results provide insight into the ANG symbiotic communities from these various hosts, the lack of methodological consistency makes meaningful conclusions about symbiont conservation across host species difficult, if not impossible. Clone libraries were generally not completed to a saturation of the community, and culture-based methods are biased towards the faster-growing and easier to culture *Gammaproteobacteria*. Both these methods would probably miss rarer taxa and make abundance-based comparisons challenging.

In this study, we investigated the conservation of the ANG community across four different cephalopod species from three cephalopod families, and from four distinct geographic ranges (Figure 1). The common cuttlefish, *Sepia officinalis*, is a member of the *Sepiidae* and is found in the eastern Atlantic from southern Scandinavia to western Africa, and throughout the Mediterranean Sea (Reid et al. 2005). The California market squid, *Doryteuthis opalescens*, a member of the *Myopsida*, is found in the eastern Pacific, ranging from Canada to the Baja Peninsula (Jereb et al. 2010). The longfin inshore squid, *Doryteuthis pealeii*, is another member of the *Myopsida* and is closely related to *D. opalescens*, but is found in the western Atlantic from Canada to Central America throughout the Gulf of Mexico and Caribbean Sea (Jereb et al. 2010). Finally, *E. scolopes*, the Hawaiian bobtail squid, is a member of the *Sepiolidae*, and is endemic to the Hawaiian archipelago (Reid and Jereb, 2005). Using next generation sequencing we were able to characterize the bacterial communities in depth and thoroughly compare composition, richness, and phylogenetic diversity. We also compared the operational taxonomic units (OTUs) present in each of the four species to determine whether any type of core community existed across species lines.

Methods

Doryteuthis pealeii ANGs (n=5) were obtained from animals collected by a collaborator at the Marine Biological Laboratory (MBL) in August of 2015 and 2016, and were maintained in our laboratory for no more than a week prior to sacrifice. *Doryteuthis opalescens* ANGs (n=2) were obtained from animals collected from Oregon (latitude 44.667, longitude -124.284) by a collaborator at Stanford University in June 2016 and were sacrificed immediately upon collection. Both *D. opalescens* animals were considered size class 4 (Lipiński and Underhill 1995), had mantle lengths of 111 and 117 mm, and had eggs present.

Sepia officinalis eggs were collected from the English Channel near Southampton, England, and were shipped to the laboratory of Roger Hanlon at the MBL. The eggs, and subsequently the growing cuttlefish, were maintained in natural seawater filtered through bioballs and kept at low temperatures (15-16°C), to keep the cuttlefish from reaching full adult size. Cuttlefish were maintained in the lab for up to 24 months and were then sacrificed and frozen and transported to UConn (n=8). Cuttlefish mantle lengths ranged from 66-88 mm.

DNA Extraction and Sequencing

DNA extraction was completed as previously described for *E. scolopes* ANGs (Kerwin and Nyholm 2017). Briefly, after the ANG tissue was surface sterilized in 99% ethanol and filter-sterilized squid Ringer's solution (FSSR, Collins et al. 2012) to remove transient bacterial contaminants, the tissue was homogenized in FSSR. Differential centrifugation was used to separate bacterial cells from the host tissue and then DNA was extracted from the bacterial component using the DNEasy Blood and Tissue kit (Qiagen, Valencia, CA) with bead-beating (Mini-Beadbeater-16, BioSpec Products, Bartlesville, OK). DNA concentration was measured using the Qubit® dsDNA High Sensitivity assay (ThermoFisher Scientific, Waltham, MA) and

averaged 34.8 ng/µl ±18.4 ng/µl (all samples > 6 ng/µl) for *S. officinalis*, was >60 ng/µl for all samples of *D. pealeii*, and was 5.61 ng/µl and 1.71 ng/µl for *D. opalescens*.

Barcoded primers developed for the V4 region of the 16S rRNA gene by Caporaso et al. (2012) were used to amplify extracted DNA. Amplified DNA was sequenced on an Illumina MiSeq (Illumina, San Diego, CA) following established protocols (Benjamino et al. 2016, Nelson et al. 2014). The UConn Microbial Analysis, Resources and Services facility (MARS) processed some of the samples.

QIIME was used to analyze sequencing data (Caporaso et al. 2010). De novo methods were used to assign OTUs at the 97% identity level (Benjamino and Graf 2016), and samples were rarified to 10,000 sequences. Alpha diversity was analyzed in QIIME, and the log2 Shannon Index was converted to a natural log Shannon Index. Alpha diversity plots were created and differences in alpha diversity between species were tested using one-way ANOVA with post-hoc Tukey tests in PRISM. Beta diversity was analyzed using the Bray-Curtis, Ochiai, and unweighted/weighted UNIFRAC metrics in QIIME, with community composition similarity tested by ANOSIM in QIIME, PCoA plots created in Microsoft Excel, and UPGMA trees created in QIIME and edited in FigTree. *S. officinalis*, *D. pealeii*, and *D. opalescens* ANG sequences were compared to *E. scolopes* sequences from Maunalua Bay, Oahu, HI, previously published and available under project ID PRJEB14655 (Kerwin and Nyholm 2017), and to *E. scolopes* sequences from Kaneohe Bay, Oahu, HI previously obtained and analyzed (Appendix I).

Results

When examining the ANG taxonomic diversity of the four host species, we first examined the bacterial classes present and found that all the communities were dominated by

Alphaproteobacteria (Figure 2). The *Alphaproteobacteria* typically made up between 50-60% of the communities, except in *S. officinalis* where they accounted for over 89% of the community on average. The remainder of the ANG communities varied between the different host species. In both *E. scolopes* and *D. pealeii*, *Gammaproteobacteria* averaged between 5-7% and *Flavobacteriia* averaged between 3-4%, but in *E. scolopes* 24% of the community was *Opitutae* (a class of *Verrucomicrobia*), while in *D. pealeii* 24% of the community was *Bacteroidia*. The *D. opalescens* ANG community contained a larger proportion of *Gammaproteobacteria* (32%) with the remaining 15% made up of *Acidimicrobiia*. *S. officinalis* was unique in not containing a substantial amount of *Gammaproteobacteria*. Instead, *Actinobacteria* made up an average of 8% of the community. At this broad taxonomic level, *E. scolopes* and *D. pealeii* appear the most similar, and *S. officinalis* the most distinct. However, this pattern breaks down when the taxonomic diversity is examined at a finer level.

The dominance by *Alphaproteobacteria* in all four species suggests the community is conserved across all lineages. However, when the *Alphaproteobacteria* taxa were examined, strong differences between the host species were visible (Figure 3). The majority of the *Alphaproteobacteria* in all four host species were *Rhodobacteraceae*, most commonly unclassified, *Leisingera* sp., and *Pseudoruegeria* sp., with the *E. scolopes Alphaproteobacteria* being almost exclusively *Rhodobacteraceae* (Figure 3A). However, the *D. pealeii Alphaproteobacteria* community was evenly split between *Rhodobacteraceae* and *Hyphomicrobiaceae*, a family which also made up most of the remaining *Alphaproteobacteria* in *D. opalescens*. Interestingly, *Inquilinus* sp., a *Rhodospirillaceae* genus, was only found in *S. officinalis,* where it accounted for almost 30% of the community. *S. officinalis* also had a larger component of *Kordiimonadaceae*, a family only seen to a lesser extent in *E. scolopes*.

When examining the other bacterial groups found in the various host species, the differences were more prominent. While both *S. officinalis* and *D. opalescens* contained bacteria from the *Actinobacteria* order, in *S. officinalis* those bacteria were exclusively from the *Pseudonocardiaceae* family (a member of the *Actinobacteria* class), while in *D. opalescens* they were from two families from the *Acidimicrobiia* class, SC3-41 and C111 (Figure 3D). *E. scolopes* had a small amount of *Bacteroidetes* present, but they were almost exclusively *Flavobacteriaceae*, while the much larger community in *D. pealeii* is mostly *Bacteroidales*, although *Arenibacter* sp., a *Flavobacteriaceae* genus was also present, as was the family *Ekhidnaceae* (Figure 3B). Finally, the *Gammaproteobacteria* were not well resolved in this analysis, with the entire *D. opalescens Gammaproteobacteria* community left unclassified to any finer level, as was most of the *D. pealeii Gammaproteobacteria* community (Figure 3C). Altogether when viewed at this level the two myopsid hosts appeared the most similar, but even these two hosts appeared quite distinct.

These findings were supported by beta diversity analysis. In four different metrics tested samples clustered by host species with very little overlap, but the clusters themselves varied in relationship to each other (Figure 4A-D). In all but the weighted UNIFRAC metric the two myopsid host clusters were closer to each other. Using Bray Curtis and weighted UNIFRAC the *E. scolopes* and *S. officinalis* clusters grouped together, but the same was not true for Ochiai or unweighted UNIFRAC. Given these patterns, the *E. scolopes* and *S. officinalis* ANG communities were more similar in terms of OTU abundance, but were more distinct from each other in terms of OTU presence and absence. The *D. opalescens* community however was more similar to that of *D. pealeii*, unless both OTU abundance and phylogenetic relationships were considered, in which case the *D. pealeii* community was distinct from all the other hosts.

In addition to the PCoA analyses, we also performed UPGMA analyses based upon the various beta diversity metrics (Figure 4E-H). The cluster patterns for Ochiai and unweighted UNIFRAC established the samples for the four different host species clustering into four monophyletic lineages, with the two myopsid hosts clustering together. The Bray Curtis metric also found the two myopsid hosts clustering together as two monophyletic lineages, but showed the *E. scolopes* samples arising from within the *S. officinalis* samples, while the weighted UNIFRAC showed the *S. officinalis* samples arising from within the *E. scolopes* cluster, along with one of the two *D. opalescens* samples.

ANG community richness, evenness, and phylogenetic diversity levels appeared similar across the various host species, with the exception of *S. officinalis*, which had lower alpha diversity in all metrics examined (Figure 5). The only other exception was of *D. opalescens* demonstrating lower levels of phylogenetic diversity than *E. scolopes* and *D. pealeii*, however this finding may be an artifact due to the low sample size examined for this host species (Figure 5C).

Samples were also examined to determine whether any core community existed across all four species. The large differences in sample size resulted in a core heavily biased towards the *E. scolopes* community (n=39) and away from that of *D. opalescens* (n=2). Two OTUs, both *Rhodobacteraceae*, were found in 95% of all samples, and accounted for an average of 44%±19% of *E. scolopes* sequences, and 50%±26% of *S. officinalis* sequences, but only for 0.02%±0.01% of *D. opalescens* sequences, and for 0.2%±0.1% of *D. pealeii* sequences.

Discussion

In this study, we compared the ANG symbiotic community of four cephalopod species from three cephalopod lineages and four distinct geographic ranges (Figure 1). While the ANG

of all four species was dominated by *Alphaproteobacteria* (Figure 2), the *Alphaproteobacteria* taxa were quite different between all four species, as were the taxa of the other major bacterial groups found (Figure 3). Beta diversity analysis revealed that while all four host species clustered independently, the two myopsid hosts, *D. pealeii* and *D. opalescens*, generally clustered more closely together (Figure 4). Similar levels of alpha diversity were found for the ANG community of *E. scolopes* and the two myopsids, while *S. officinalis* had lower overall alpha diversity.

Sepia officinalis

The *S. officinalis* community composition found in our analyses was similar in some ways to previously published research, but we also found several distinct differences. Using light microscopy Grigioni et al. (2000) found that the majority of the *S. officinalis* community was Gram positive, a result we cannot confirm in this study. Using a combination of FISH and clone libraries, members of the community were found to include the *Alphaproteobacteria* genera *Agrobacterium* and *Rhodobium-Xanthobacter* (both from the *Rhizobiales* order) and *Roseobacter* (from the *Rhodobacteraceae*), while Gram positive groups included an *Actinobacteria* genus, the *Sporichthya*, and a member of the *Firmicutes* phylum, *Clostridium* (Grigioni et al. 2000). By contrast, members of the *Rhizobiales* order made up less than 0.3% of the average community in this study (Figure 3A). Our research does demonstrate that Gram positive bacteria account for a portion of the *S. officinalis* community, but in another disparity, they made up on average only 8% of the community and were exclusively from the *Pseudonocardiaceae* family (Figure 3D). These differences could be due to a zoo effect and the lack of the natural environmental bacterial community necessary for horizontal symbiont transmission. Alternatively, we could have experienced difficulties extracting DNA from Gram positive organisms and sequencing DNA

with the high GC content common in the *Actinobacteria*. While our protocols should work well for Gram positive bacteria, future work on adult animals collected from the wild will be necessary to conclusively determine the ANG community of *S. officinalis*.

Many of the differences noted between *S. officinalis* and the other host species included in this analysis, such as *Alphaproteobacteria* accounting for such a high proportion of the community (Figure 2) and the lower levels of alpha diversity in all *S. officinalis* ANGs (Figure 5), could be due to these animals being raised in captivity, i.e. a zoo effect. Unlike the other host species examined here, the *S. officinalis* ANGs were collected from animals raised in a laboratory setting, in filtered natural seawater, and were prevented from growing to full adult size by maintaining the animals at lower temperatures. As the ANG community is thought to be environmentally transmitted, the composition of ANGs from animals raised in the lab may not be comparable to wild-caught hosts. Previous research on lab-raised *E. scolopes* animals has found unusually high levels of *Alphaproteobacteria* in animals which did develop an ANG (Chapter 4). While raising *S. officinalis* in the lab did not appear to prevent ANG development, the ANG bacteria normally found in the environment of this host may not have been present in the lab environment, and thus were not available to seed the community. Additionally, the stunted growth of the animals could indicate that the ANG community had not yet reached its mature state, potentially explaining the lower alpha diversity found (Chapter 4).

Doryteuthis pealeii and *D. opalescens*

Much of the previous research into the ANG community of myopsid squid has been culture-based, and as a result isolates obtained from these squid have generally been members of the *Gammaproteobacteria* (Barbieri et al. 1996, Lum-Kong and Hastings 1992). Research utilizing FISH and clone libraries concluded that the bacterial community of *D. pealeii* was 65%

Alphaproteobacteria and 5% *Gammaproteobacteria* (Barbieri et al. 2001), a finding identical to the data presented here. CFB strains have also been found in clone libraries from loliginids (Barbieri et al. 2001, Pichon et al. 2005), a finding also confirmed by our results. To our knowledge, the *D. opalescens* ANG has not been previously examined for taxonomic diversity. However, most myopsid ANGs have been found to contain members of the *Rhodobacteraceae*, *Gammaproteobacteria*, and CFBs, although in all cases only a few clones were obtained per species (Pichon et al. 2005). We found significant differences between the two myopsids examined here in terms of both relative abundance and presence/absence of certain groups (*Gammaproteobacteria*, *Bacteroidales*, and *Acidimicrobiia*), although the various *Alphaproteobacteria* taxa were similar between the two host species. The low sample size examined for *D. opalescens* makes drawing firm conclusions about alpha and beta diversity difficult, but the ANG communities of these two host species appeared to be similar to each other in terms of alpha diversity and to be more similar to each other taxonomically than to the other hosts examined here.

Overall patterns in diversity

This study sequenced the ANG bacterial community for four species from three different cephalopod families and four different geographic regions. While we saw some similarities in the two myopsid species, such as the *Alphaproteobacteria* composition, no other pattern was especially evident based on host species phylogeny. Geographic patterns were also difficult to discern in this dataset. *Verrucomicrobia* was found only in the Pacific hosts, while *Actinobacteria* only made up a substantial portion of the community in the Atlantic hosts. The other potential trend may be latitudinal, in which case we would expect to see the myopsids grouping with *S. officinalis* to the exclusion of *E. scolopes*. This result was obtained only through

the use of the unweighted UNIFRAC beta diversity metric (Figure 4H). In the future, adding additional host species from other cephalopod groups which have different geographic distributions, some of which overlap, may make the detection of patterns easier. Additionally, increasing the sample size for *D. opalescens* and collecting wild adult *S. officinalis* will strengthen this analysis. These additions will also make the computation of a core community more informative.

The diversity of the ANG bacterial community across host species is striking, especially given the potentially crucial functional role of this consortium in egg protection (Chapter 4). The lack of vertical transmission in this system could explain the variation to a degree, as various host species adapt to the local bacterial consortia present in their environments. While *E. scolopes* maintains a diurnal cycle, burying under the sand during the day, *S. officinalis* live on sand and mudflats but do not bury, and myopsid hosts live entirely pelagic lives, only utilizing the benthos to deposit their eggs. These different lifestyle patterns may influence the bacteria available for environmental transmission and could explain some of the variation observed between the hosts. The stark differences in geographic ranges could also lead to varying environmental threats for the different hosts. Predation pressure is known to be highest around the equator, and to decrease at higher latitudes (Vermeij 1978), and symbiotic chemical defenses which are present in southern bryozoan species, are lacking in northern species (McGovern and Hellberg 2003). While not much is known about varying fungal load in different geographic locations across the oceans, *Fusarium keratoplasticum* FSSC-2g, the fungal pathogen described in Chapter 3, does not germinate in temperatures under 15°C (personal obs.), and would probably not threaten cephalopod eggs in colder waters, although other fungi could be potential pathogens. Finally, functional redundancy between bacterial members of the various host ANG

communities could mean that similar protection is given to the various embryos by very different bacterial species.

While the presence of the ANG symbiosis is conserved across many cephalopod lineages, the composition of the ANG bacterial community does not appear to be strongly conserved. The exception to this finding is the strong presence of *Rhodobacteraceae* in all host ANGs examined. The *Rhodobacteraceae* are an important marine bacterial family which can account for up to 25% of certain marine microbial communities, and are especially abundant in coastal waters where ANG-containing cephalopods are found (Wagner-Döbler and Biebl 2006). Members of this clade have been found in symbiosis with a number of marine hosts including oysters (Ruiz-Ponte et al. 1998), algae (Rao et al. 2007), and sponges (Zan et al. 2014). Roseobacter *Leisingera* sp. JC1, a member of the *E. scolopes* ANG community, has been shown to produce secondary metabolites which inhibit certain marine vibrios (Gromek et al. 2016), suggesting that the *Rhodobacteraceae* could play an important role in egg defense in all ANG-containing cephalopods.

The ANG was first described over 100 years ago in *D. pealeii* (Williams 1909), and only recently has the function of the community been described (Chapter 3). Recently the bacterial community of the *E. scolopes* ANG has come under investigation as a novel source of anti-fungal drug discovery. Characterizing the ANG communities of other cephalopod hosts could potentially provide a much wider pool of candidates for these drug discovery efforts. The diverse bacteria from these varying ANG communities may play a protective role against other as yet unexplored pathogens and biofoulers. The dramatic differences reported here for the various host species demonstrates the remarkable diversity of marine bacteria that can become symbiotic partners and potentially play similar roles in egg defense across various cephalopod hosts.

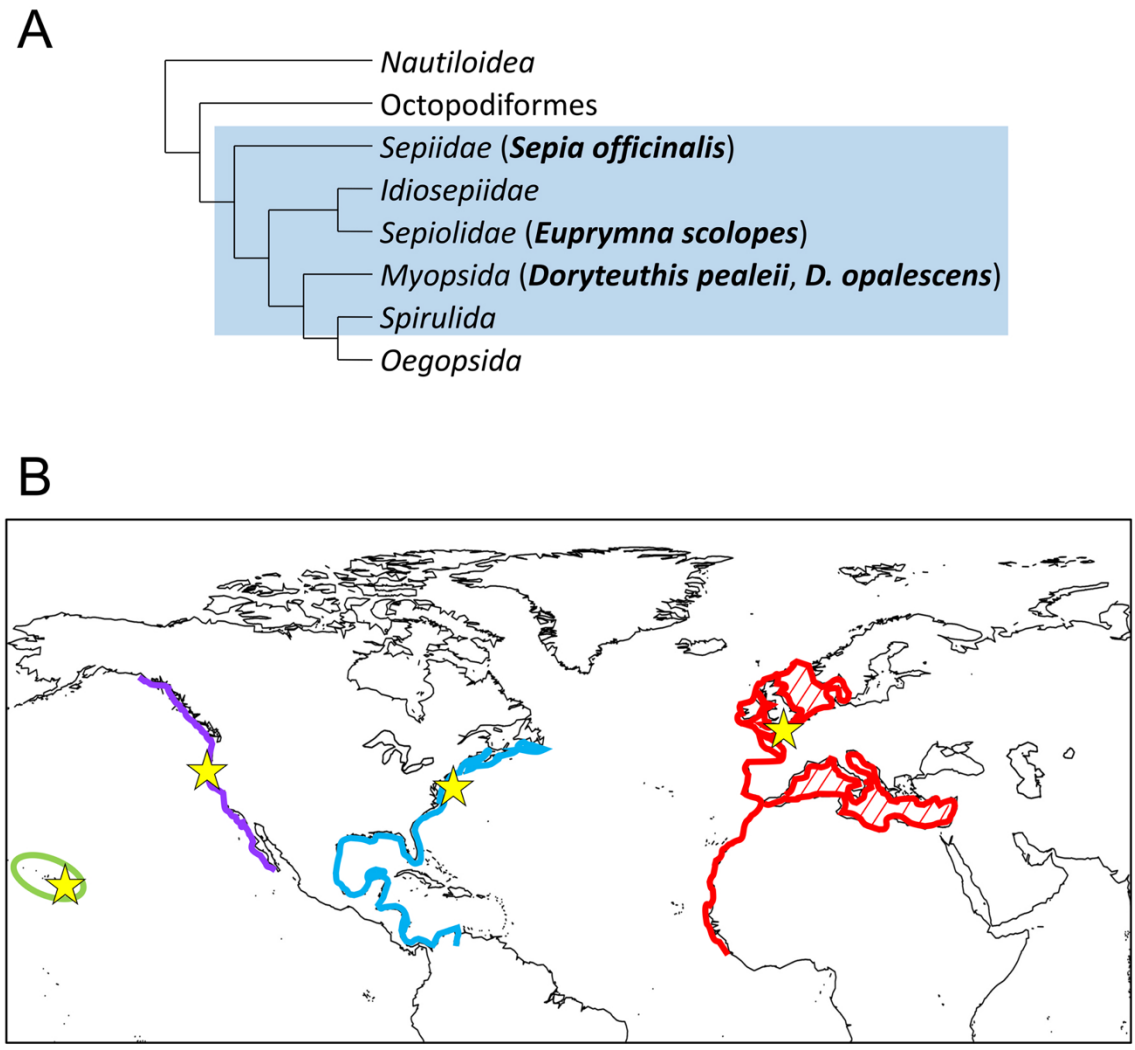


Figure 1. ANGs are known to be present in five cephalopod lineages (A, blue box). Our study examines the ANG communities of four species (A, bolded text), from four geographic ranges (B, green – *E. scolopes*, purple – *D. opalescens*, blue – *D. pealeii*, red – *S. officinalis*). Yellow star indicates site of animal collection within each range (egg collection for *S. officinalis*). Phylogeny adapted from Tanner et al. 2017. Ranges adapted from Jereb et al. 2010, Reid et al. 2005, and Reid and Jereb 2005.

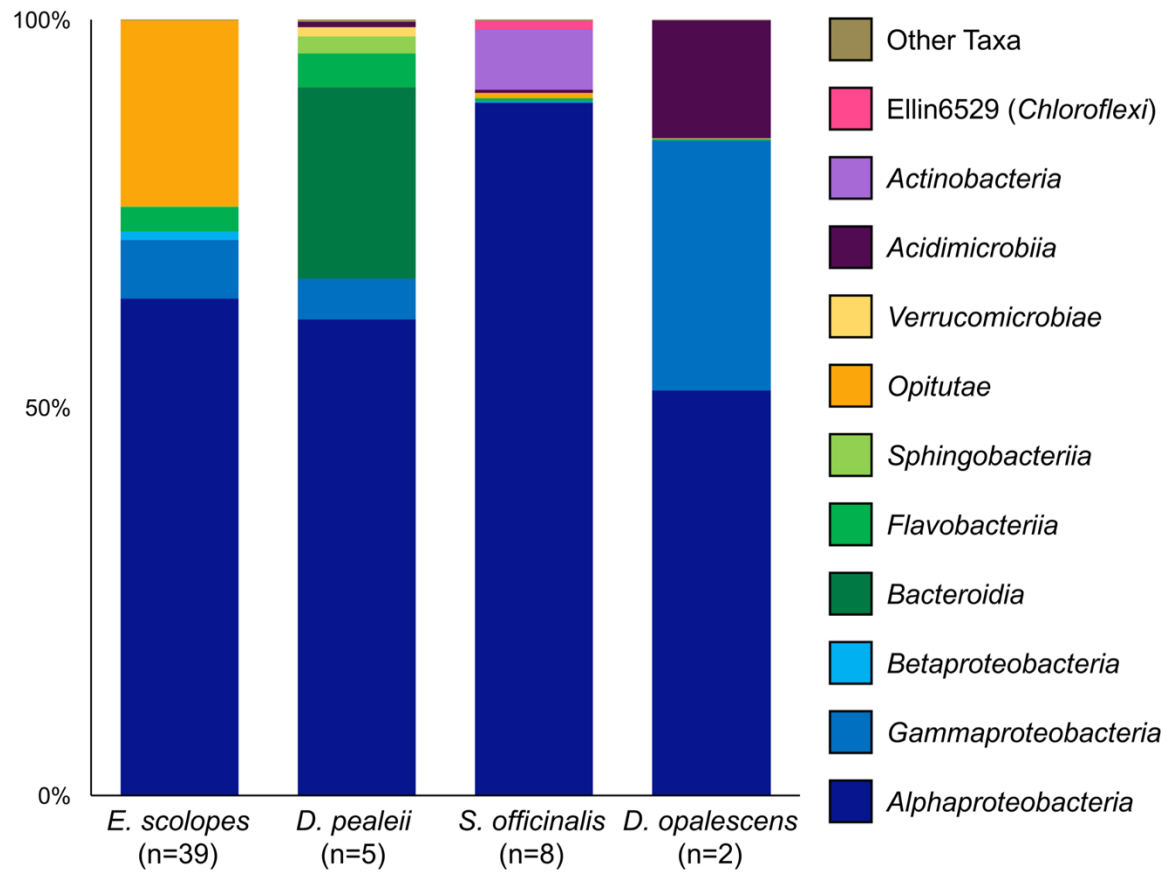


Figure 2. ANG taxonomic diversity of four cephalopod species. Average relative abundance per species presented at the class level.

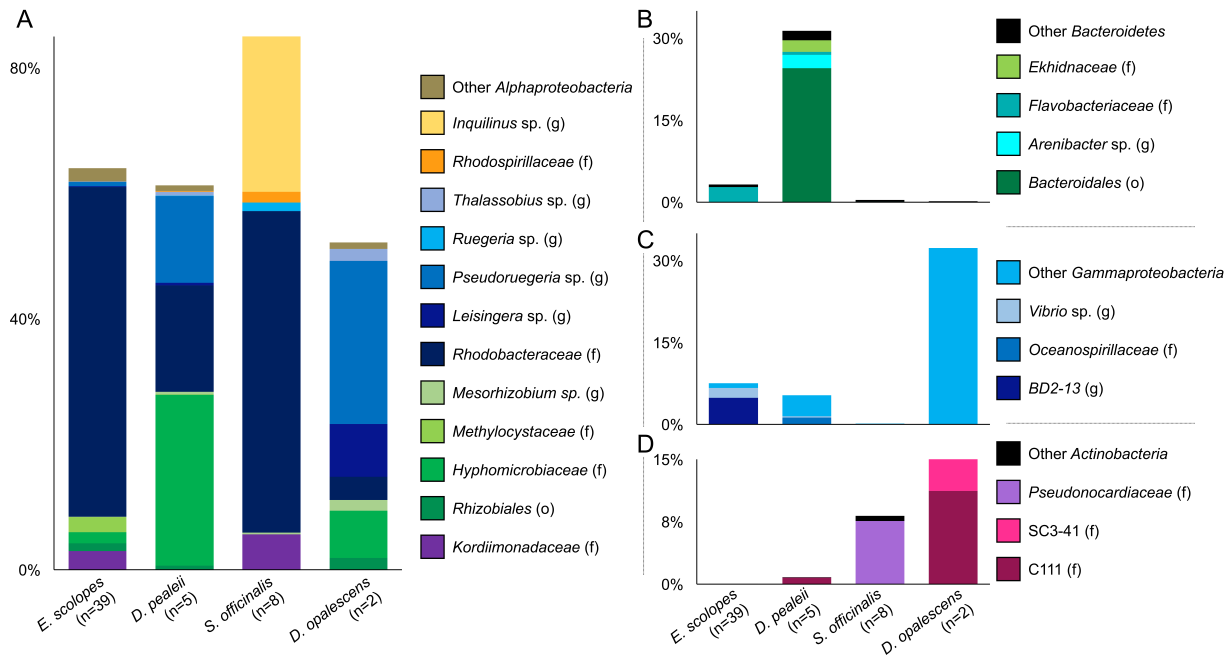


Figure 3. ANG taxonomic diversity of four cephalopod species separated by bacterial group into lineages from the *Alphaproteobacteria* class (A), *Bacteroidetes* phylum (B), *Gammaproteobacteria* class (C), and *Actinobacteria* phylum (D). Average relative abundance per host species presented at the finest taxonomic level obtained (c – class, o – order, f – family, g – genus).

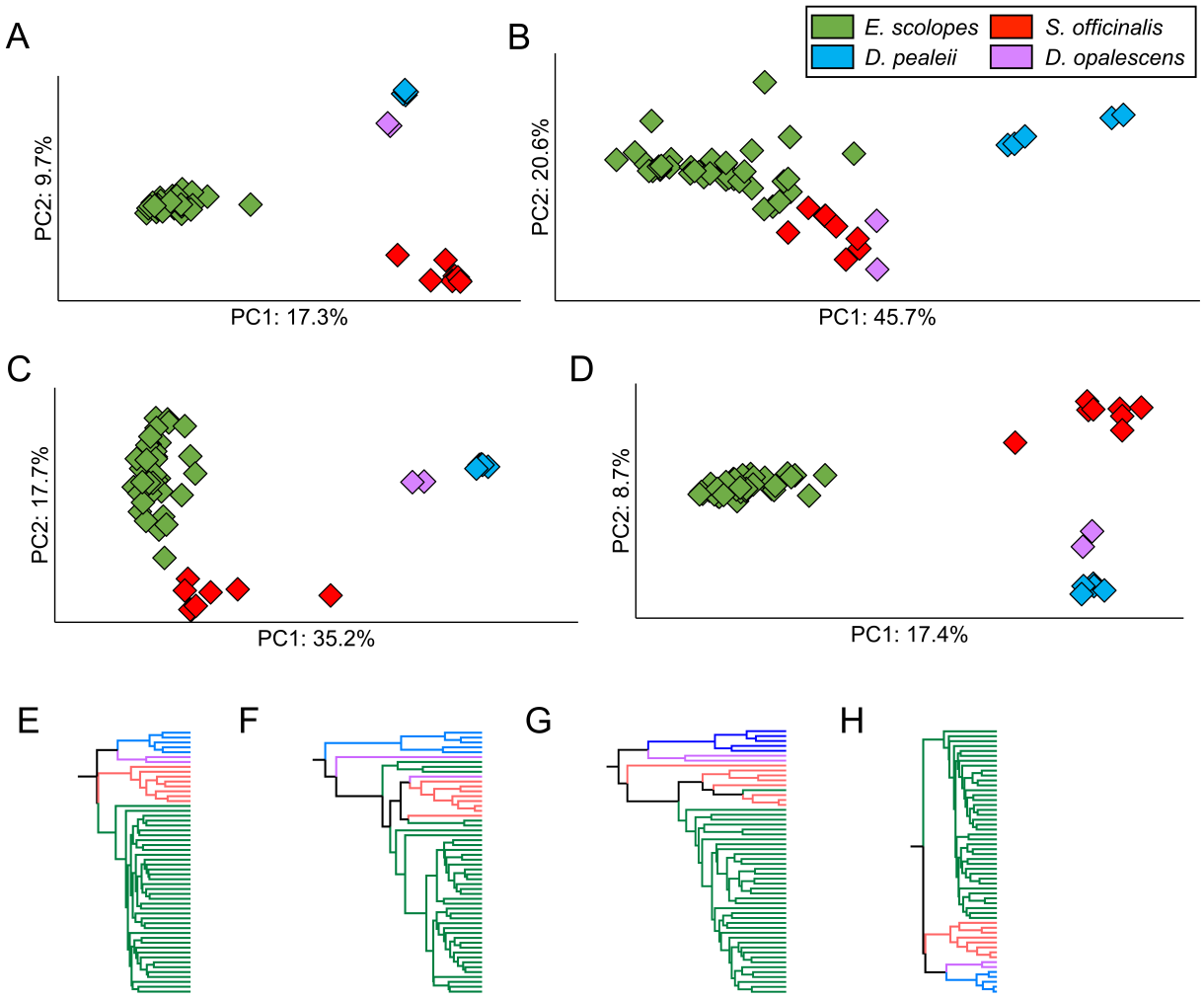


Figure 4. Beta diversity analyses plotted on PCoA plots show that the four species contain distinct communities which cluster apart from each other (A-D). Beta diversity analyses plotted as UPGMA trees showed similar patterns for two metrics (E,H), while two other metrics showed *S. officinalis* and *E. scolopes* communities interspersed with each other (F,G). Metrics tested include Ochiai (A,E), weighted UNIFRAC (B,F), Bray Curtis (C,G), and unweighted UNIFRAC (D,H).

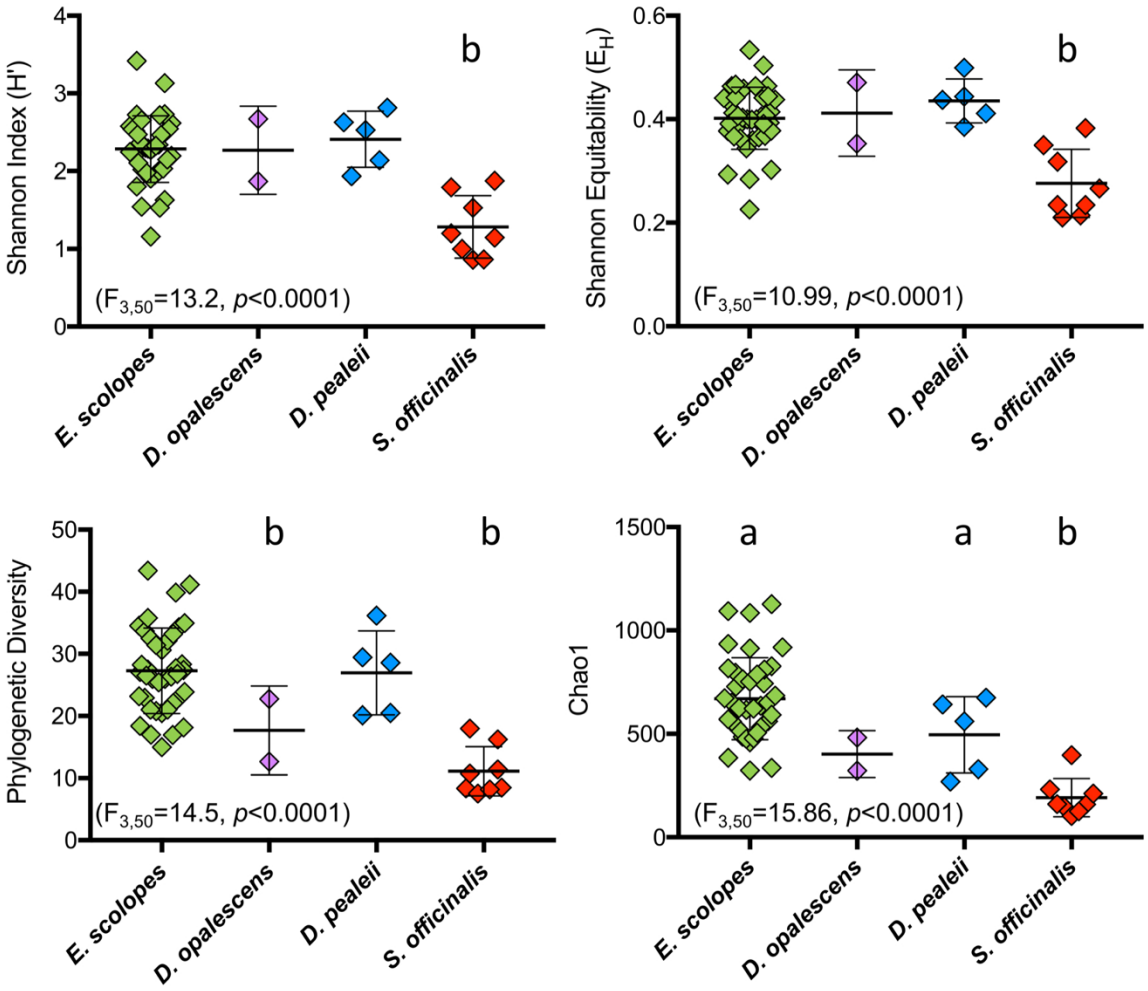


Figure 5. Alpha diversity metrics demonstrate that the *S. officinalis* community has lower richness, evenness, and phylogenetic diversity than the ANG communities of the other species, possibly due to these animals being lab-raised. Thick bars indicate mean and thin bars indicate standard deviation. One-way ANOVA results displayed in parentheses, *post-hoc* Tukey test results displayed as letters above significantly different host species.

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