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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

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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.

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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

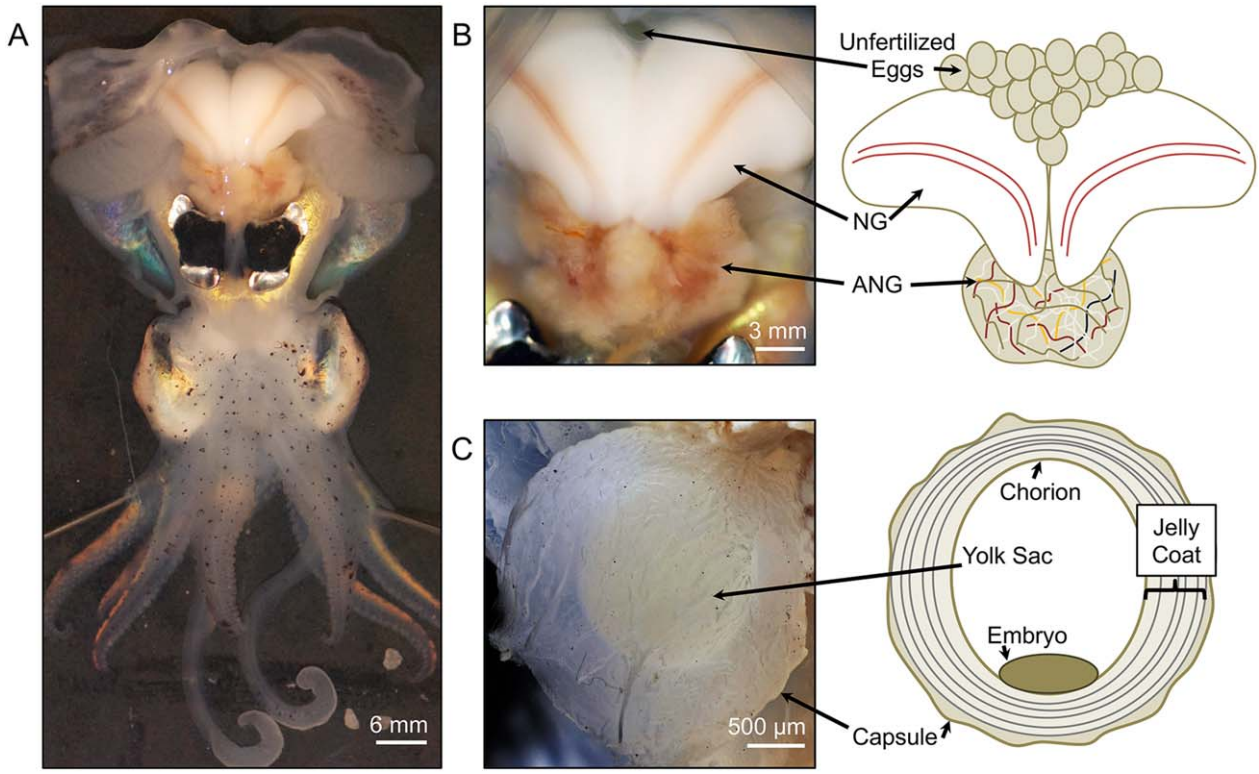


Fig. 1. Ventral dissection of a female *E. scolopes* (A), the female reproductive system (B) and an egg (C). Bacteria from the ANG are deposited into the JC layers that are secreted by the nidamental glands (NG). The embryo develops inside the central yolk sac, bounded by the chorion membrane. The JC 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.

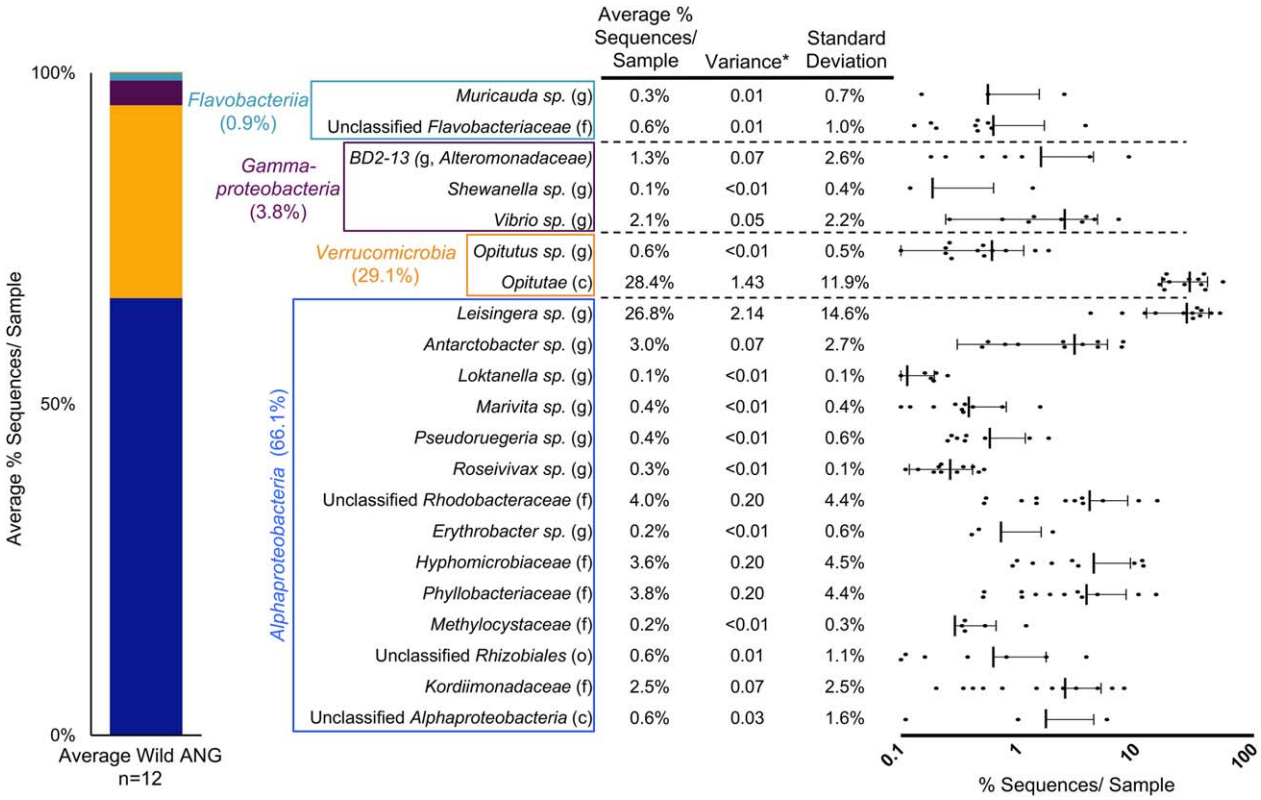


Fig. 2. The average symbiont population in the ANG of mature, wild-caught animals was composed of four bacterial classes. The percentage of sequences per sample for each taxon did not vary widely indicating a consistent community composition throughout the population of *E. scolopes*. Taxa present in 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

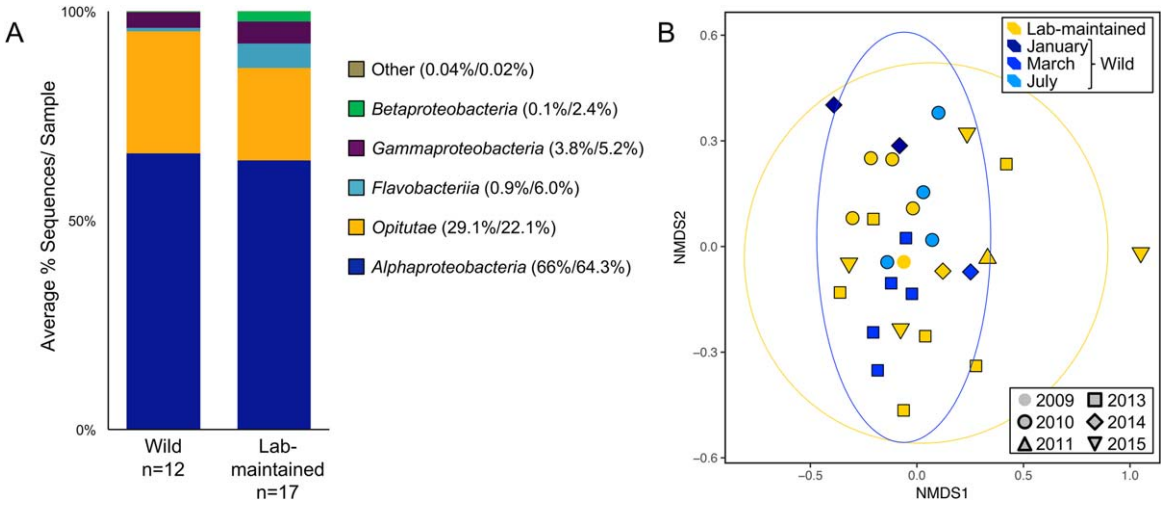


Fig. 3. Wild ($n = 12$) and lab-maintained ($n = 17$) ANGs had similar bacterial community compositions, both in the most abundant bacterial classes (A) and in beta-diversity (B). Bray Curtis NMDS plot of the two types of communities shows overlap of the sample types indicating that the community composition 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.

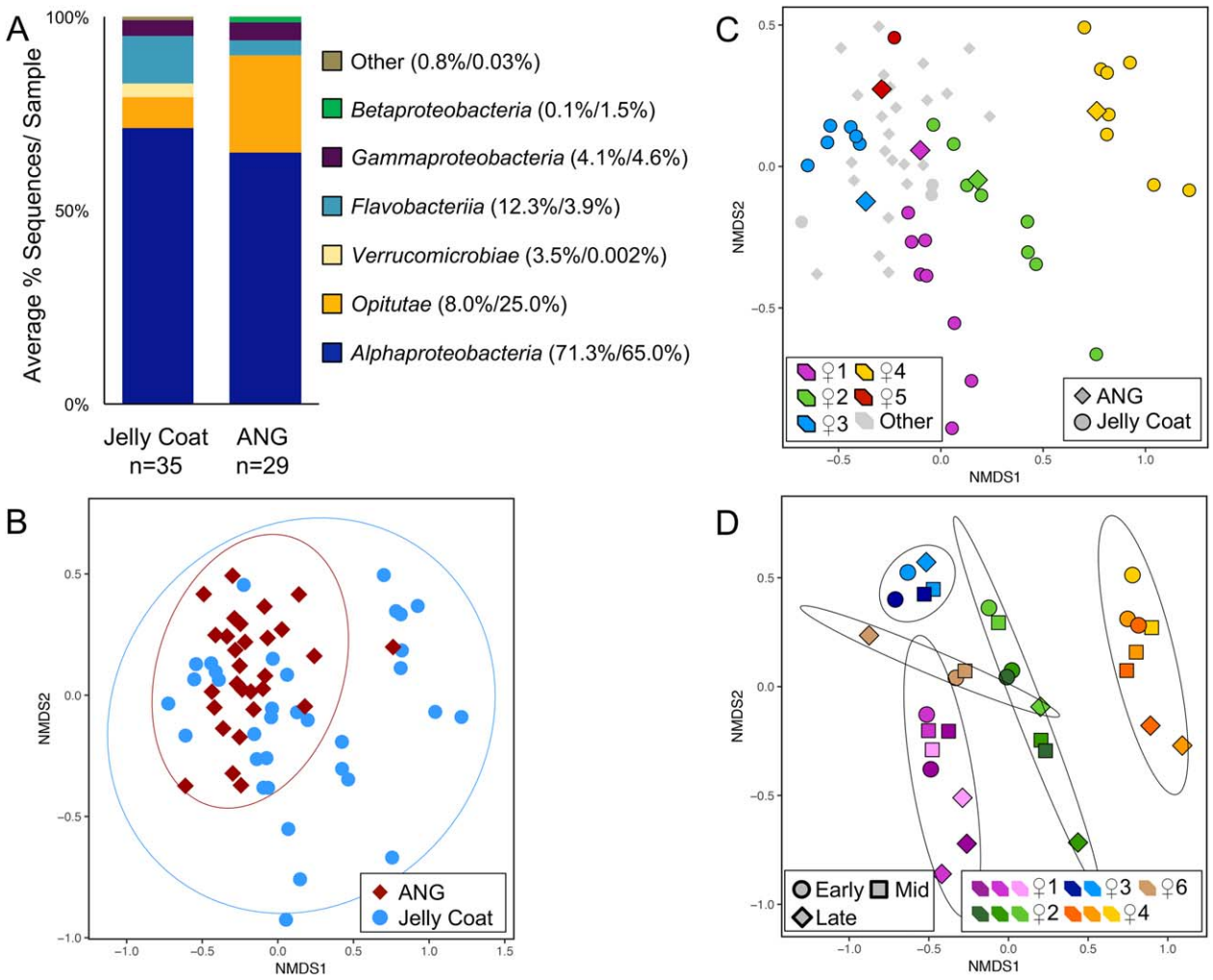
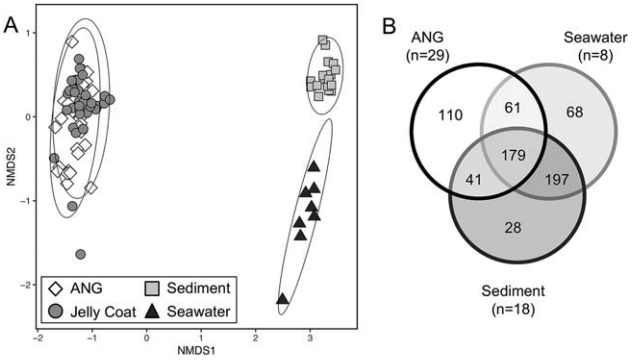


Fig. 4. The egg JC ($n = 35$) and ANG ($n = 29$) had similar bacterial community compositions, consisting of 4 main bacterial taxa (A). The 'other' component included taxa present in more than one sample, and at less than 0.5% of the average community composition. Other taxa made up 0.8% of the JC community 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

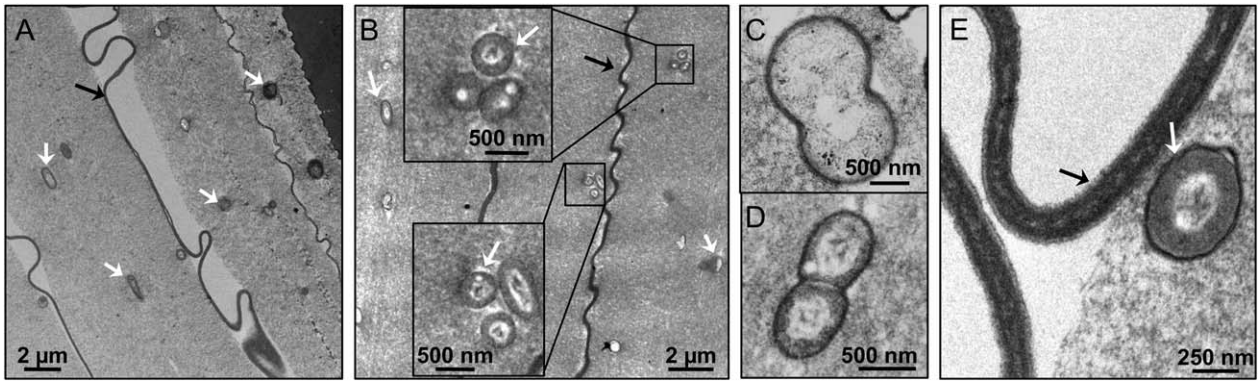


Fig. 6. Transmission electron micrographs of *E. scolopes* egg JCs collected at day 0 (A) or day 21 (B) of embryogenesis. At day 0 bacteria occurred as single cells throughout the various layers of JC (A), while at day 21 small microcolonies of bacteria of similar morphologies were common (B, see insets). 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
Proteobacteria	*Alphaproteobacteria*	*Kordiimonadales*	*Kordiimonadaceae*		7	1.34%
		Rhizobiales	Unclassified *Rhizobiales*		1	0.05%
			Phyllobacteriaceae		1	3.01%
		Rhodobacterales	*Rhodobacteraceae*	Unclassified *Rhodobacteraceae*	17	15.56%
				Leisingera sp.	7	29.30%
				Loktanella sp.	1	0.08%
				Marivita sp.	1	0.34%
				Roseivivax sp.	1	0.14%
				Antarctobacter sp.	4	5.51%
Verrucomicrobia	*Opitutae*	Unclassified *Opitutae*			8	23.18%
		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.

Fig. S5. Laboratory-maintained *E. scolopes* were kept in captivity for periods ranging from two weeks to four months.

Fig. S6. DNA extractions yielded a variety of concentrations, but the majority if not all of the replicates for most sample types were $>$1ng/μl (A). The exception to this cutoff were the JC samples, five of which were below this cutoff but which appeared similar in composition to others that were sequenced, and the lab substrate and lab seawater samples, sequenced as controls. All included samples yielded $>$ 10,000 sequences, with the exception of one lab seawater sample, one JC sample, and one Hawaiian sediment sample, all of which yielded 5,000-10,000 sequences (B).

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.