frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 09 December 2014
doi: 10.3389/fmicb.2014.00593

Microbial experimental evolution as a novel research approach in the Vibrionaceae and squid-*Vibrio* symbiosis

***William Soto*[1] *and Michele K. Nishiguchi*[2] ***

[1] *BEACON Center for the Study of Evolution in Action, Michigan State University, East Lansing, MI, USA*
[2] *Department of Biology, New Mexico State University, Las Cruces, NM, USA*

Edited by:
Monica Medina, Pennsylvania State University, USA

Reviewed by:
Natacha Kremer, Université Claude Bernard Lyon 1, France
Catherine Masson-Boivin, Institut National de la Recherche Agronomique, France

***Correspondence:**
Michele K. Nishiguchi, Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Foster Hall- Horseshoe and Sweet Streets, Las Cruces, NM 88003-8001, USA
e-mail: nish@nmsu.edu

The Vibrionaceae are a genetically and metabolically diverse family living in aquatic habitats with a great propensity toward developing interactions with eukaryotic microbial and multicellular hosts (as either commensals, pathogens, and mutualists). The Vibrionaceae frequently possess a life history cycle where bacteria are attached to a host in one phase and then another where they are free from their host as either part of the bacterioplankton or adhered to solid substrates such as marine sediment, riverbeds, lakebeds, or floating particulate debris. These two stages in their life history exert quite distinct and separate selection pressures. When bound to solid substrates or to host cells, the Vibrionaceae can also exist as complex biofilms. The association between bioluminescent *Vibrio* spp. and sepiolid squids (Cephalopoda: Sepiolidae) is an experimentally tractable model to study bacteria and animal host interactions, since the symbionts and squid hosts can be maintained in the laboratory independently of one another. The bacteria can be grown in pure culture and the squid hosts raised gnotobiotically with sterile light organs. The partnership between free-living *Vibrio* symbionts and axenic squid hatchlings emerging from eggs must be renewed every generation of the cephalopod host. Thus, symbiotic bacteria and animal host can each be studied alone and together in union. Despite virtues provided by the Vibrionaceae and sepiolid squid-*Vibrio* symbiosis, these assets to evolutionary biology have yet to be fully utilized for microbial experimental evolution. Experimental evolution studies already completed are reviewed, along with exploratory topics for future study.

Keywords: *Vibrio*, sepiolid squid, cospeciation, experimental evolution, environmental transmission

THE VIBRIONACEAE

The family Vibrionaceae (Domain Bacteria, Phylum Proteobacteria, Class Gammaproteobacteria) encompass gram-negative chemoorganotrophs that are mostly motile and possess at least one polar flagellum (Farmer III and Janda, 2005; Thompson and Swings, 2006); although, the gut symbiont *Vibrio halioticoli* to the abalone *Haliotis discus hannai* has been described as non-motile (Sawabe et al., 1998). Vibrionaceae are facultative anaerobes, having both respiratory (aerobic and anaerobic) and fermentative metabolisms. Nitrogen fixation and phototrophy have both been reported (Criminger et al., 2007; Wang et al., 2012). Agarases and alginases have been noted from *Vibrio* (Fu and Kim, 2010; Dalia et al., 2014). Most cells are oxidase positive with a dimension 1 μm in width and 2–3 μm in length. Sodium cations are a requirement for growth and survival, but *Vibrio cholerae* and *V. mimicus* are unusually tolerant to low sodium waters. Most species are susceptible to the vibriostatic agent 0/129 (Thompson and Swings, 2006). Vibrionaceae are ubiquitously distributed throughout aquatic habitats, including freshwater, brackish, and marine waters (Madigan and Martinko, 2006). Vibrionaceae have been isolated from rivers, estuaries, lakes, coastal and pelagic oceanic waters, the deep sea, and saltern ponds (Urakawa and Rivera, 2006). Vibrionaceae can also be microbial residents of aquatic animals as either commensals, pathogens, and mutualists (Soto et al., 2010). Bacteria may exist as planktonic free-living cells or as biofilms attached to solid subtrates present in sediments of aquatic habitats or alternatively adhered to floating particulate matter or debris. Vibrionaceae may also form biofilms on the surfaces of animal, algal/phytoplanktonic, protoctistal, or fungal hosts the cells colonize, as this prokaryotic family is quite able to initiate and establish vigorous biofilms on eukaryotic cells and chitin surfaces (e.g., invertebrate exoskeletons and fungal cell walls; Polz et al., 2006, Pruzzo et al., 2008; Soto et al., 2014). Vibrionaceae have also been found to be intracellular inhabitants of eukaryotic microorganisms (Abd et al., 2007). Although as many as eight genera have been assigned to the Vibrionaceae, the two most specious are *Vibrio* and *Photobacterium* (Thompson and Swings, 2006). *Salinivibrio* possesses an unusual ability to grow in a wide range of salinity (0–20% NaCl) and temperature (5–50°C; Ventosa, 2005; Bartlett, 2006). Numerous species in the Vibrionaceae are pathogenic and cause disease in aquatic animals and humans (Farmer III et al., 2005), *V. cholerae* being the most notorious example as the causative agent of cholera (Colwell, 2006). *V. vulnificus* and *V. parahaemolyticus* can also cause severe illnesses in humans as a result of consuming contaminated seafood (Hulsmann et al., 2003; Wong and Wang, 2004). Furthermore, every year *V. harveyi* (Owens and Busico-Salcedo, 2006), *V. anguillarum*

(Miyamoto and Eguchi, 1997; Crosa et al., 2006), and *V. parahaemolyticus* (Austin, 2006) cause substantial economic losses to the aquaculture industry worldwide. The genera *Vibrio* and *Photobacterium* include opportunistic pathogens capable of infecting marine animals and humans, and are able to enter preexisting wounds or body openings of especially susceptible hosts that are already ill, stressed, fatigued, or immunocompromised (Urbanczyk et al., 2011). Given the heightened ability of Vibrionaceae to cement themselves to eukaryotic cells through peptide and polysaccharide modification of their exopolysaccharide, lipopolysaccharide, and capsules (Sozhamannan and Yildiz, 2011), the lack of additional human pathogens is curious. Perhaps the reason is foreign extracellular protein and polysaccharide are the same materials the mammalian immune system specifically targets, neutralizes, and removes as non-self antigens with exquisite capacity (Owen et al., 2013). Vibrionaceae have also been recently investigated for the development of probiotics, antimicrobials, and pharmaceutical drugs with potential clinical and economic value for veterinary medicine, animal husbandry, aquaculture, and human health—molecules antagonistic toward cancer cells, fungi, algae, protoctists (a term frequently preferred over protist or protozoan), bacteria, and viruses. Metabolites produced by the Vibrionaceae have also been found to have quorum sensing-disrupting properties against other bacteria, which may open an entire horizon for the advancement of "quorum sensing" antibiotics (i.e., quorum quenching) (Gatesoupe, 1999; Mansson et al., 2011).

MICROBIAL EXPERIMENTAL EVOLUTION

Conventional evolutionary studies seeking to understand adaptation and speciation implement the comparative or historical approach (e.g., phylogenetics). This approach compares organisms from different environments and attempts to understand the evolutionary processes that may have produced the current distributions and adaptations of descendent populations from ancestral ones (Bennett, 2002). Since this methodology generates informed explanations based on extant organisms retrospectively and with hindsight, it naturally must make numerous assumptions on the evolutionary relationships of the organisms under study and their likely mode of evolution, even when the use of fossil data is available. Experimental evolution, however, allows one to begin with an ancestral population and empirically observe the adaptation and radiation that result in the descendent lineages under different selective regimens. Experimental evolution studies can be implemented under controlled and reproducible conditions to study evolution, usually in the laboratory and on model organisms. Less assumptions in environmental conditions, the selection pressures involved, or in the ancestral and evolving populations are necessary, since there is more control by the investigator (Bennett, 2002). Experimental evolution permits tractability for the study of evolutionary biology by allowing experiments to be manipulated and repeated with replication (Lenski, 1995; Bennett, 2002). Bacteria, including *Vibrio*, are ideal organisms for such studies. For instance, these organisms have short generation times which allow evolution and adaptation to be observable on a human time scale (Lenski, 1995). Microorganisms also usually possess the advantage of achieving large population sizes ($>1 \times 10^9$ cells/mL in liquid culture) in the environments for which experimental evolution studies are executed, providing ample opportunity for rare beneficial mutations to arise and reach fixation by natural selection. Deleterious mutations are likely to become extinct via purifying selection, since evolution by genetic drift is negligible in huge gene pools. Moreover, a "frozen fossil record" can be generated with bacteria by storing evolving lineages at different evolutionary time points in a −80°C freezer. Hence, one can later compare relative fitness of the ancestral clone with a derived one in novel or ancestral environments (Bennett, 2002; Lenski, 2002). As a result, evolutionary tradeoffs can be measured during the course of adaptation in the novel environment. The −80°C fossil record also permits the determination of the evolutionary episode that a novel adaptive trait first evolved. Likewise, evolution may be "replayed" from various time points to see if subsequent outcomes are contingent on prior genetic changes or previously modified traits (Kawecki et al., 2012; Barrick and Lenski, 2013). Finally, the ancestral and derived bacteria can subsequently be analyzed to observe the exact genetic changes that have occurred and which specific ones are responsible for novel adaptive traits (Lenski, 1995; Bennett, 2002). Experimental evolution is the only direct method for studying adaptation and the genetic changes responsible, which complements genetic, physiological, biochemical, and phylogenetic approaches.

ATTENUATION AND VACCINE DEVELOPMENT WITH VIBRIOS: INSIGHTS FOR MICROBIAL EXPERIMENTAL EVOLUTION

Microbial experimental evolution is a thrilling sub-discipline of evolutionary biology which has risen in the last twenty to thirty years to address diverse issues (Soto et al., 2010; Conrad et al., 2011). Although initial work largely began with *Escherichia coli* (Lenski et al., 1991), the inclusion of other microbial species has continued to grow. However, despite a few exceptions (Schuster et al., 2010; Soto et al., 2012, 2014), surprisingly little work has been completed to date with members of the Vibrionaceae. Considering the Vibrionaceae possess colossal metabolic, biochemical, ecological, and genetic diversity, the general absence of this bacterial family as an established model in microbial experimental evolution has been heedless. Nonetheless, classical efforts to attenuate pathogenic bacteria for human vaccine development were endeavors analogous to experimental evolution (Kawecki et al., 2012). Virulent bacterial isolates would be repeatedly subcultured under laboratory conditions on growth medium, in tissue/cell culture, or in animal models to introduce random deleterious mutations in the microorganism under study. Alternatively, the microbe would be continuously subjected to chemical or physical mutagens (e.g., ultraviolet light). The exact mutations that occurred and the loci undergoing genetic changes were frequently unknown initially, and attempts to their identification only coming later with additional research (Frey, 2007). For *V. cholerae*, nitrosoguanidine frequently served as a chemical mutagen to induce several attenuating mutations, including auxotrophy (Bhaskaran and Sinha, 1967; Baselski et al., 1978). Although attenuation by random mutagenesis yielded some products that demonstrated promising results in animal models and humans, this approach is less common today (Frey,

2007). The construction of attenuated vibrios containing large targeted deletions of loci known to contribute to virulence is currently more desirable, since microbial reversion to pathogenicity is deemed less probable through this practice. Side effects are also a concern (Honda and Finkelstein, 1979; Cameron and Mekalanos, 2011). *V. cholerae* attenuation by the continual introduction of random mutations, resulting in numerous deleterious genetic lesions across many loci, frequently fails to sufficiently incapacitate virulence (Cameron and Mekalanos, 2011), as the microbe finds alternative ways to thrive and persist in the human host. An evolutionary conclusion coming from vaccine work with *V. cholerae* is that numerous ways of making a successful living exist in a potential host for the genus *Vibrio*; many potential niches exist, as evidenced by the continued ability of *V. cholerae* to initiate successful and alternative symptomatic infections (e.g., reactogenicity) despite the introduction of several deleterious mutations into its genome. An implication of this observation is that vibrios are evolutionarily versatile for host colonization and proliferation. For instance, medical reports exist of *V. cholerae*'s ability to initiate bacteremia, malaise, fever, chills, and skin lesions in humans, even in the absence of a gastrointestinal infection (Ninin et al., 2000). Such symptoms are more typically characteristic of *V. vulnificus* infections and raise the interesting question of whether there may be common virulence factors in *V. cholerae* and other pathogenic vibrios which are overshadowed by the exuberant effect of choleragen. More broadly, determinants and mechanisms responsible for the colonization of host animals (or attachment to eukaryotic cells) by vibrios may possess overlap across diverse interactions (e.g., commensalism, pathogenesis, and mutualism; Hentschel et al., 2000). Hence, microbial selection experiments with vibrios have potential to provide novel insights into evolution of the varied interactions the genus *Vibrio* possesses with its hosts, and vibrio vaccine research is a great repository of information and useful starting point to ask scientific questions, construct hypotheses, and to find focus topics for real world applications and practical value.

SEPIOLID SQUID-*VIBRIO* SYMBIOSIS: A CASE STUDY FOR MICROBIAL EXPERIMENTAL EVOLUTION WITH THE VIBRIONACEAE

As mentioned previously, many members of the Vibrionaceae are able to form associations with eukaryotic hosts, including phytoplankton, protoctists, algae, aquatic fungi, invertebrates, fishes, and aquatic mammals, which may range from harmful, neutral, and beneficial to the host (Soto et al., 2010; Urbanczyk et al., 2011). One particular mutualistic interaction is the partnership between marine bioluminescent *Vibrio* and sepiolid squid. The sepiolid squid-*Vibrio* symbiosis has been a model system for studying developmental biology, immunology, physiology, and molecular biology underpinning interactions between bacteria and animals for over two decades (McFall-Ngai and Ruby, 1991), since both partners can easily be maintained in the laboratory independently of each other. Sepiolid and loliginid squids (**Figure 1A**) are colonized by bioluminescent *Vibrio* (Fidopiastis et al., 1998; Guerrero-Ferreira and Nishiguchi, 2007). The bioluminescent bacteria inhabit a morphological structure called the light organ (**Figure 1B**) within the squid mantle cavity and benefit from their association with the cephalopod host by inhabiting a microenvironment rich in nutrients relative to the oceanic water column. The squid hosts prosper from the presence of bioluminescent bacteria by utilizing the light produced for a cryptic behavior called counterillumination (Jones and Nishiguchi, 2004; **Figure 1C**). Female squid fertilized by males lay their eggs on solid substrates such as rocks, where the embryos develop. Since female sepiolid squids are sequential egg layers, they can produce several clutches over 4–5 months after sexual maturity, with each clutch being 50–500 eggs each (Moltschaniwskyj, 2004). Axenic squid hatchlings emerge from their eggs (usually at twilight or at night) with sterile light organs, which are colonized within a few hours by specific free-living bioluminescent *Vibrio* present in the ocean (Soto et al., 2012). The colonizing bacteria quickly reproduce to fill and occupy the light organ crypt spaces (i.e., lumina, **Figure 1D**). Daily at dawn 90–95% of the light organ symbionts are vented exteriorly to the ocean by the squid host prior to burying in the sand. The remaining bacterial fraction in the squid host re-grows throughout the day to reinstate a complete light organ population by sunset (Soto et al., 2012). At dusk the squid emerge from the sand to engage in their nocturnal activity, including foraging and mating. [More detailed and comprehensive information can be found in recent reviews (Dunn, 2012; Stabb and Visick, 2013)]. Since bioluminescent symbionts can be grown in pure culture, cryopreserved with possible subsequent resuscitation, genetically manipulated and analyzed, and used to inoculate recently hatched gnotobiotic squid juveniles, the sepiolid squid-*Vibrio* mutualism is a promising prospect for experimental evolution studies aiming to understand symbioses. The juvenile squids are born without their *Vibrio* symbionts, lending the ability to infect the juveniles with any strain of *Vibrio* bacteria to examine colonization rates, ability to colonize, and persistence. Additionally, these bacteria can be used in competition experiments, which allows one to test different wild type strains against one another, mutant strains against their original wild type strain or versus other mutants, and experimentally evolved strains against the original ancestor (Nishiguchi et al., 1998; Nishiguchi, 2000, 2002; Soto et al., 2012).

Nevertheless, this mutualism has only recently been tapped as a resource for microbial experimental evolution studies in recent years (Schuster et al., 2010; Soto et al., 2012, 2014). Early work has shown *V. fischeri* are able to adapt to a novel squid host within 400 generations (**Table 1**), and such evolution may create tradeoffs in the ancestral animal host environment or in the free-living phase as a physiological correlated response to an important abiotic factor (Soto et al., 2012). Two sepiolid squid genera, *Euprymna* and *Sepiola*, are in the same taxonomic family. Several different *Euprymna* species are distributed allopatrically throughout the Indo-West Pacific Ocean, while numerous *Sepiola* species simultaneously co-occur sympatrically in the Mediterranean Sea (Nishiguchi et al., 1998; Nishiguchi, 2000, 2002; Soto et al., 2009). *Vibrio* symbionts colonizing *Euprymna* are host specialists and outcompete allochthonous isolates, a phenomenon termed competitive dominance, while those colonizing *Sepiola* are host generalists. *Vibrio* symbionts display no competitive dominance within *Sepiola* (Nishiguchi et al., 1998; Nishiguchi, 2000, 2002; Wollenberg

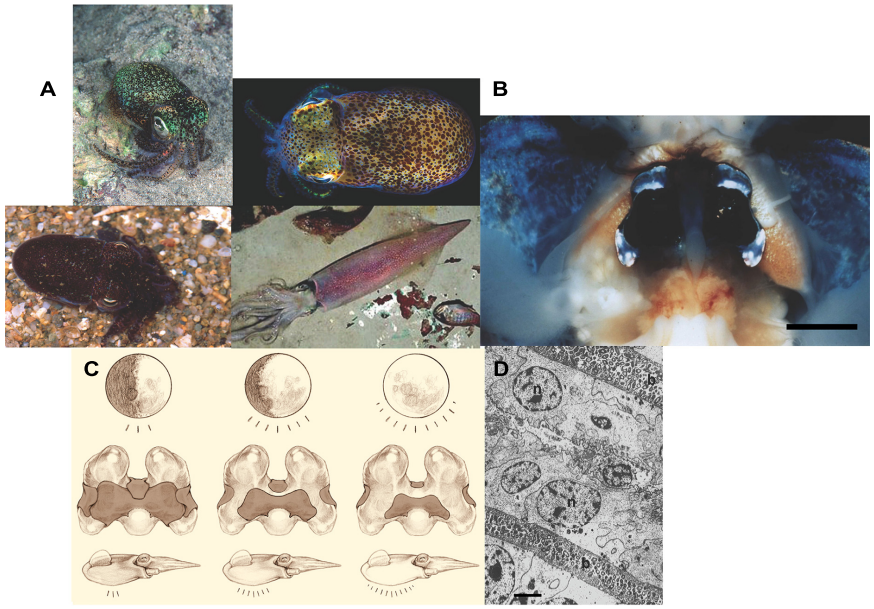


FIGURE 1 | The sepiolid squid-*Vibrio* symbiosis. (A) Representative species from the families Sepiolidae and Loliginidae (clockwise from upper left): *Euprymna tasmanica* (Sepiolidae), *E. scolopes* (Sepiolidae), *Photololigo noctiluca* (Loliginidae), and *Sepiola affinis* (Sepiolidae). **(B)** Ventral dissection of *E. scolopes*, showing the bilobed light organ surrounded by the ink sac. Bar = 0.5 cm. **(C)** Diagram how the light organ operates under different phases of the moon. The progressive decrease in shading from left to right symbolizes increased illumination by the light organ. **(D)** A transmission electron micrograph of an area of the epithelium-lined crypts containing symbiotic bacteria: (n) = nucleus of squid cell, (b) = bacteria in crypts (bar = 10 μm). Photo credits: Mark Norman, Mattias Oremstedt (Kahikai), M. K. Nishiguchi, R. Young, S. Nyholm, R. Long, M. Montgomery. Light organ illustration by Robert Long-Nearsight graphics.

Table 1 | Competitive colonization experiments between *Vibrio fischeri* strains ES114 (ancestor) and JRM200 (derived) at different evolutionary time points in the novel squid host *Euprymna tasmanica*.

Evolutionary time point (Generations)	Expected ES114:JRM200 (Percentage ancestor: percentage evolved)	Observed ES114:JRM200 (Percentage ancestor: percentage evolved)
0 (n = 33)	50:50	46:54
100 (n = 24)	50:50	47:53
200 (n = 24)	50:50	41:59
300 (n = 24)	50:50	41:59
400 (n = 24)	50:50	35:65[1]
500 (n = 24)	50:50	36:64[1]

[1] *Significantly different two-tailed t-test and sign test ($P < 0.05$, $\alpha = 0.05$).*

and Ruby, 2012). Despite the presence of competitive dominance, data from population genetics and phylogenetics suggested secondary colonization events have occurred (Nishiguchi and Nair, 2003; Jones et al., 2006; Urbanczyk et al., 2011), creating a puzzling conundrum for years. Population genetics surveys fueled this enigma by consistently observing high levels of genetic diversity within the squid light organ (Jones et al., 2006), indicating light organ populations are not dominated by single or few genotypes through space and evolutionary time, an observation not consistent with competitive dominance. Competitive dominance results from squid host specialization by the symbionts, which should presumably purge genetic diversity of *V. fischeri* populations inside light organs. Microbial experimental evolution shed light on these mysteries and helped resolve these paradoxes with a complementing temporal population genetics survey spanning a decade—about 20,000 *V. fischeri* generations of evolution within the squid host—revealed the same evolutionary forces begetting competitive dominance were also responsible for driving *V. fischeri* genetic and phenotypic diversity within the squid light organ (Soto et al., 2012, 2014). *V. fischeri* indigenous to the Hawaiian bobtail squid (*E. scolopes*) was serially transferred for 500 generations through the Australian dumpling squid (*E. tasmanica*), a novel host (Soto et al., 2012; **Figure 2**).

Results demonstrated as *V. fischeri* adapted to *E. tasmanica*, the ability of the derived lines to grow along a salinity gradient significantly changed relative to the ancestor. Moreover, no obvious pattern to the growth changes was evident across the salinity continuum, suggesting *V. fischeri* microbial physiology had been "randomized." Salinity is known to impact *Vibrio* population levels and distributions worldwide (Soto et al., 2009). *V. fischeri* subjected to novel host evolution created polymorphic reaction norms for salinity, an abiotic factor integral to shaping symbiont ecology during the free-living phase. Furthermore, experiments indicated a "superior numbers" or a "running start" advantage to foreign strains over native ones in animal host colonization that could outflank competitive dominance. Thus, *V. fischeri* strains most abundant (perhaps due to salinity) during the free-living phase where squid hosts resided were the ones most likely to colonize

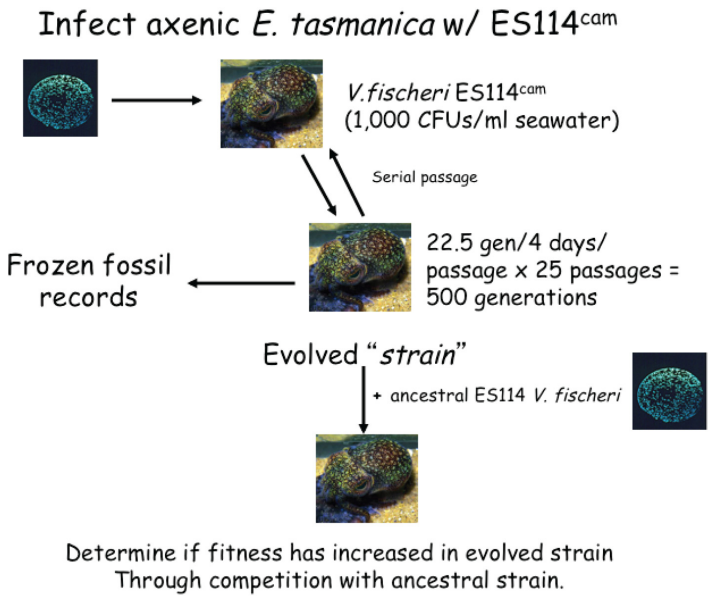


FIGURE 2 | Flow chart of experimental evolution studies in the Southern dumpling squid, *E. tasmanica*. *Vibrio fischeri* ES114^{CAM} is a isogenic strain of *V. fischeri* ES114 (native to the Hawaiian bobtail squid, *E. scolopes*), with a neutral chloramphenicol-resistance marker (CAM). This strain was passaged through a non-native host for 500 generations to determine whether fitness would improve over each generation. Throughout the study, frozen fossil records can be made to eventually compare ancestor to evolved clones. Similar experiments can be completed in *E. scolopes*, including control animals (Soto et al., 2012).

the cephalopod, not strains best adapted to the squid (Soto et al., 2012). A similar process may occur with *Photobacterium* in fish hosts due to temperature (Urbanczyk et al., 2011). Additionally, the *V. fischeri* lines serially passaged through *E. tasmanica* surged in biofilm formation and bioluminescence but lessened in motility (Soto et al., 2014). Increases and decreases in the utilization of select carbon sources also transpired. Interestingly, evolutionary differentiation occurred in the derived lines relative to the ancestor and to each other for biofilm formation, motility, bioluminescence, and carbon source metabolism, results consistent when compared to *V. fischeri* wild isolates obtained from light organs of *E. scolopes* and *E. tasmanica* specimens collected in the field (Soto et al., 2014). Squid host specialization by the symbionts promotes competitive dominance and diversifying selection. Perhaps clonal interference prevents selective sweeps in the squid light organ. The lineages serially transferred through *E. tasmanica* also exhibited decreased levels of bioluminescence in the ancestral host *E. scolopes* (Soto et al., 2012). In an independent study, *V. fischeri* strains previously incapable of establishing a persistent association (chronic infection) with sepiolid squids were shown to be capable of doing so after serial passage in *E. scolopes* (Schuster et al., 2010). Since *V. fischeri* possesses a life history where bacteria are cyclically associated with an animal host (sepiolid squids and monocentrid fishes) and then outside the host as free-living bacteria in the ocean, researchers can use microbial selection experiments with *V. fischeri* to simultaneously study symbiosis evolution and microbial evolution in the natural environment where microbes are not partnered to a host. [Some recent work suggests *V. fischeri* may also be a bioluminescent symbiont in the light organs of fishes belonging to the taxonomical families Moridae and Macrouridae (Urbanczyk et al., 2011).] Additionally, *V. fischeri* strains exist which are completely unable to colonize the light organs of sepiolid squids and monocentrid fishes, permitting evolutionary biologists to study a continuum of interactions between a microbe and animal host when studying the squid-*Vibrio* mutualism. Given the Sepiolidae is a diverse family of squids that include allopatric and sympatric species distributions, testing whether host speciation affects selection for host specialist versus host generalist evolutionary strategies within *Vibrio* symbionts is possible.

TYPE STRAIN MENTALITY AND OTHER BIOLUMINESCENT SYMBIONTS FOR SEPIOLID SQUIDS

Early work characterizing the molecular biology of *V. fischeri* colonizing *Euprymna* squid focused on the strain *V. fischeri* ES114 and the host *E. scolopes* (with occasional studies in *Sepiola*), since only the Hawaiian squid host was routinely available (McFall-Ngai and Ruby, 1991; Fidopiastis et al., 1998). Furthermore, reductionism was desired to understand the fundamentals of the symbiosis. Nonetheless, caution is warranted to avoid development of a "type strain" or "type host" mentality. Recent work has expanded to regularly include other strains of *V. fischeri* and *Euprymna* species (Ariyakumar and Nishiguchi, 2009; Chavez-Dozal et al., 2012; Soto et al., 2012). This will aid in identifying more general results from those that are specific to a particular symbiont strain or host species. In addition, initial characterization of the sepiolid squid-*Vibrio* symbiosis described *V. fischeri* as the only bioluminescent symbiont present in the squid light organ (McFall-Ngai and Ruby, 1991). Subsequently, *V. logei* was discovered as a symbiont in the genus *Sepiola* (Fidopiastis et al., 1998;

Nishiguchi, 2000). More recently, *V. harveyi* and *Photobacterium leiognathi* have been included as symbionts of *E. hyllebergi* and *E. albatrossae* from Thailand and the Philippines, respectively (Guerrero-Ferreira and Nishiguchi, 2007; Guerrero-Ferreira et al., 2013). An important prospect to consider is that *V. fischeri* and *V. logei* may have evolved fundamentally distinct and different traits for colonizing sepiolid squids, even when considering the same host species. Clearly, new and thrilling perspectives are surfacing around the sepiolid squid-*Vibrio* mutualism. Several species in the Vibrionaceae are bioluminescent. An interesting remaining question is why only a few of these form light organ symbioses with sepiolid squid hosts. For example, why is bioluminescent *V. orientalis* never found in squid light organs (Dunlap, 2009)? Are researchers simply not looking thoroughly enough?

BIOGEOGRAPHY OF *VIBRIO* BACTERIA AND EXPERIMENTAL EVOLUTION IN THE FIELD

Experimental evolution in the lab with *Vibrio* bacteria has only been completed in one species of *Vibrio* (*V. fischeri*), and strains used in those studies were either from the squid host *E. scolopes* (Hawaii) or pinecone fish *Monocentris japonicas* (Schuster et al., 2010; Soto et al., 2012). Given that a number of symbiotic *V. fischeri* from squid can colonize and survive in nearly all allopatric *Euprymna* hosts of the Indo-West Pacific, it provides a road map whether other *V. fischeri* strains can adapt to additional potential host species closely related to *Euprymna* (e.g., *Rondeletiola minor*) or even ones from a different phylum (Nishiguchi et al., 2004). Naturally occurring strains may be subjected to movement between hosts that are along a specific environmental gradient (Soto et al., 2010). Obviously, similar cues must be used for these bacteria to recognize a comparable, yet novel host, and then colonize and establish a persistent association in the outré animal for the symbionts to secure their distribution in the new host population (Wollenberg and Ruby, 2009). Only 6–12 *V. fischeri* cells are required to initiate a squid light organ infection. Once these bacteria colonize a squid host, they can reproduce much faster than in seawater. New *V. fischeri* clones encountering a squid host species for the first time will then be expelled every 24 h, increasing the cell numbers of *V. fischeri* new arrivals that can infect even more juvenile squid of the exotic host species (Lee and Ruby, 1994b, 1995). Whether these symbiont founder flushes truly occur in nature is not known, but observations in the laboratory have shown that alien *V. fischeri* genotypes can invade and take root where a preexisting genetic variety was already entrenched (Lee and Ruby, 1994a; Soto et al., 2012). Whether this commonly leads to a dominant symbiont genotype in a host population in a given geographical area over the long term must be investigated more closely.

TWO-CHROMOSOME GENOMIC ARCHITECTURE IN VIBRIONACEAE, EVOLVABILITY, AND VERSATILITY

Research has shown an absence of parallel coevolution between *V. fischeri* symbionts and their light organ animal hosts, which implies significant host switching has occurred (Nishiguchi and Nair, 2003). Host switching has been a common evolutionary phenomenon for *Vibrio* and *Photobacterium* species involved in symbioses, regardless of whether the interaction was commensalism, pathogenesis, or mutualism (Urbanczyk et al., 2011). Extensive host switching could suggest this microbe, along with *Vibrio* species in general, are evolutionarily plastic and malleable organisms. Vibrionaceae possess two circular chromosomes, one large (Chromosome I) and one small (Chromosome II; Tagomori et al., 2002). With this complex genome arrangement, *V. fischeri*'s ability to exploit numerous lifestyles is easy to understand, as the *Vibrio* genome structure is dynamically unstable (Kolsto, 1999). The modular two-chromosome architectural structure of Vibrionaceae genomes has been hypothesized to be the inception for the versatility and ubiquity of this cosmopolitan bacterial family, with ecological specialization being the essence of the smaller and more genetically diverse Chromosome II with its superintegron island gene-capture system and genes encoding for solute transport and chemotaxis (Heidelberg et al., 2000; Ruby et al., 2005; Grimes et al., 2009). Intrachromosomal and interchromosomal recombination is clearly present, along with inversions, indels, and rearrangements (Kolsto, 1999; Heidelberg et al., 2000; Tagomori et al., 2002). Such genomic architecture permits the evolutionary potential for functional genetic specialization to occur among the two chromosomes (Heidelberg et al., 2000; Waldor and RayChaudhuri, 2000), promoting ecological opportunity in adapting and radiating into numerous niches (Soto et al., 2014). For example, *V. cholerae* and *V. parahaemolyticus* genomic studies have discovered that house-keeping genes (DNA replication, transcription, translation, cell division, and cell wall synthesis) and pathogenicity are mainly restricted to the large chromosome (Heidelberg et al., 2000).

Chromosome II appears to be a genetic module for DNA and a source for innovation, perhaps evolutionarily functioning analogous to plasmids, possessing significantly more foreign loci that appear to have been acquired horizontally from other microbial taxa (Heidelberg et al., 2000; Waldor and RayChaudhuri, 2000). The presence of a gene capture system (i.e., integron island) and genes usually found on plasmids support this claim (Heidelberg et al., 2000). Furthermore, loci involved in substrate transport, energy metabolism, two-component signal transduction, and DNA repair are prominently carried on Chromosome II (Heidelberg et al., 2000; Waldor and RayChaudhuri, 2000). The loci involved in substrate transport consist of a large repertoire of proteins with diverse substrate specificity. Genes that subdivide cellular functions and that are intermediaries of metabolic pathways also are found on Chromosome II. These genetic auxiliaries potentially serve as the raw material for adaptation and specialization (Heidelberg et al., 2000; Waldor and RayChaudhuri, 2000). The structure and size of the large chromosome appears relatively constant throughout the Vibrionaceae, whereas Chromosome II is more acquiescent and flexible to genetic reorganization, rearrangement, recombination, and large indel events (Okada et al., 2005). Genes encoding function for starvation survival and quorum sensing are located on both chromosomes. Thus, interchromosomal functional regulation is present in Vibrionaceae. As a result, specific and novel mechanisms involved in the regulation, replication,

and segregation of both chromosomes are thought to have evolved in this bacterial family (Waldor and RayChaudhuri, 2000; Egan et al., 2005).

Interestingly, *V. cholerae* colonization factors (e.g., genes responsible for pili formation) primarily reside on Chromosome I. Consequently, different *V. fischeri* ecotypes could be the result of evolution at loci involved in metabolism as opposed to those involved in tissue colonization (Browne-Silva and Nishiguchi, 2007; Soto et al., 2014). Experimental evolution studies with *E. coli* have demonstrated that resource partitioning and alternative substrate specialization is sufficient for ecological polymorphisms to arise in prokaryotes (Rosenzweig et al., 1994). In summary, the two-chromosome architecture provides *V. fischeri* with enormous evolutionary fluidity. Particularly, Chromosome II may possess ecological or symbiosis islands which could account for this microorganism's broad ecological range (Tagomori et al., 2002). For example, differences in the pathogenicity islands present on Chromosome II appear to determine whether or not *V. parahaemolyticus* strains are pathogenic. Similarly, the pliant nature of *V. fischeri* could explain why there is extensive host switching. Chromosome II may well be a gene repository outfitted to respond to environmental change, habitat heterogeneity through space and time, and stress (Dryselius et al., 2007; Soto et al., 2012). Future studies will be thrilling and exciting, as modern bioinformatics and genomics offer high hopes and allow unprecedented visions. Recent advances in high throughput sequencing technologies and genome editing techniques (e.g., MuGENT) will greatly increase the potential of experimental evolution to understand adaptation (MacLean et al., 2009; Dalia et al., 2014).

TOPICS FOR FUTURE STUDY

BIOFILM FORMATION AND MOTILITY

Motility and biofilms are modes by which *V. fischeri* strains can niche specialize in their *Euprymna* squid hosts (Yildiz and Visick, 2009; Soto et al., 2014). Biofilms are aggregates of microorganisms attached to a surface that are frequently enmeshed within a matrix of exopolysaccharide and can be comprised of a pure culture population or a community (Davey and O'toole, 2000; Stoodley et al., 2002). This community is much more resistant to antimicrobials, ultraviolet light, pH shifts, osmotic shock, desiccation, and other environmental stresses (Gilbert et al., 1997; Davey and O'toole, 2000). The role of biofilms in disease and host colonization is well documented, where bacterial pathogens establishing biofilms in animals may be more recalcitrant to phagocytosis by host macrophages, resistant to respiratory bursts by immune cells, and insensitive to antimicrobials produced by host defenses (Davey and O'toole, 2000). In addition, biofilm development has a major role in *V. fischeri* colonization of sepiolid squid hosts (Chavez-Dozal and Nishiguchi, 2011; Chavez-Dozal et al., 2012; **Figure 3**). When movement on surfaces is necessary, swarming with flagella is the motility mechanism for Vibrionaceae (McCarter, 2001). Swarming is specialized mobilization or locomotion on a surface as opposed to the swimming and tumbling done by individual cells. As *V. fischeri* swarms with concurrent cell division (e.g., growth), cells differentiate from a vegetative state to a swarmer one. Swarmer cells are hyperflagellated and longer than vegetative counterparts (Harshey, 2003), and provide a steady state supply of nutrients until motility ceases. Motility plays an integral role in the colonization of sepiolid squid by *V. fischeri* and allows host-associated bacteria to reach the destination and surface desired for further colonization or attachment (Millikan and Ruby, 2004). Since swarming is an energetically expensive process, chemotaxis has a role mediating how a bacterial cell should physiologically respond. Through

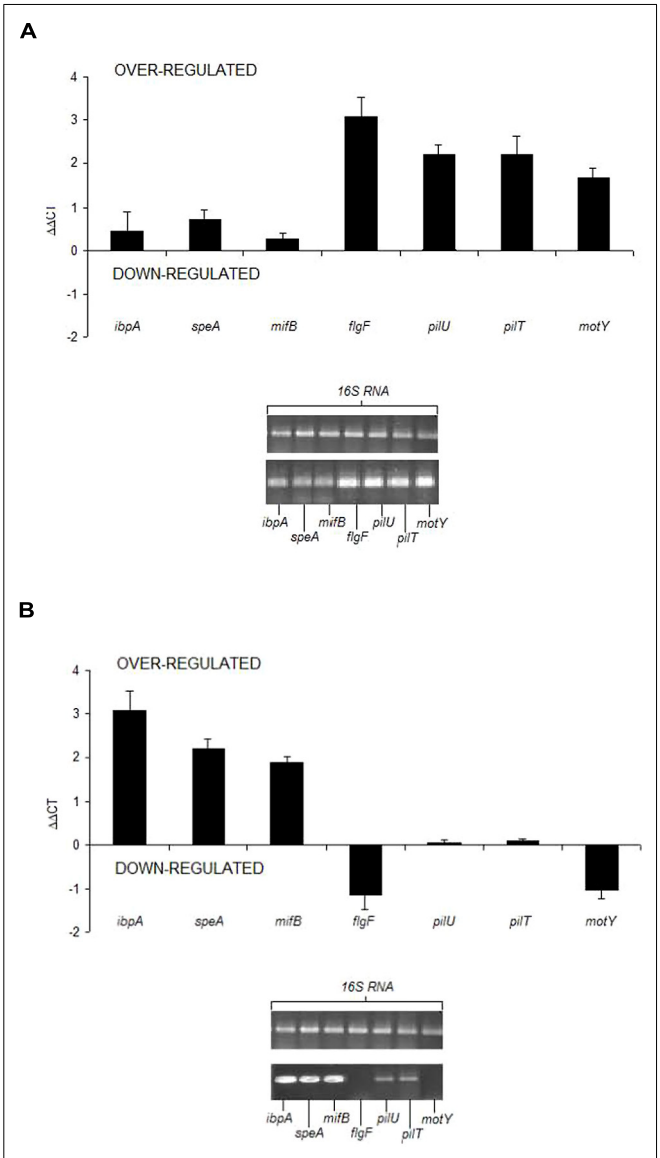


FIGURE 3 | Early and late gene expression (mRNA) of various biofilm related loci in *V. fischeri* ETJB1H. (A) Early (4 h) gene expression of flagellum biosynthesis (*flgF*), type IV pili formation and adhesion (*pilU, pilT*), and the sodium-type flagellar motor pump for motility (*motY*) loci. **(B)** Late gene expression of genes important for mature biofilm production (24 h) include expression of heat shock protein (*ibpA*), magnesium-dependent induction for c-di-GMP synthesis (*mifB*), and arginine decarboxylase (*speA*). Modified from Chavez-Dozal et al. (2012). dCCT is the change in relative expression of each gene compared to the standard control (in this case 16S rRNA). The gel represents the amount of 16S rRNA (top) and the amount of mRNA expressed in each gene examined. Error bars represent SD of three replicates.

years of studying diverse bacteria as motility model systems, research has shown many regulatory pathways controlling motility also affect biofilm formation (Harshey, 2003; Verstraeten et al., 2008). Bacterial populations must resolve whether to institute motile machinery for expedient colonization of surfaces or engage biofilm systems when an appropriate location for initial contact and attachment has been found, a critical choice affecting survival between competitors. Experiments are underway where *V. fischeri* lines are being selected for increased biofilm formation and motility. Accompanying these experiments are ones where *V. fischeri* lines are being alternately or cyclically selected for biofilm and motility lifestyles (oscillatory selection). The relative abilities of these lines to colonize squid hosts will be assessed.

PARASITISM, PREDATION, AND GRAZING ON *VIBRIO* BACTERIA

Substantial work exists on how protoctistan predators are effective grazers on *Vibrio* or other bacteria, particularly when they form biofilms (Matz and Kjelleberg, 2005; Matz et al., 2008). Previous research has demonstrated that certain species of *Vibrio* (e.g., *V. cholerae* and *V. fischeri*) are better able to ward off microbial eukaryotic predators when in their biofilm state compared to their planktonic counterparts (Erken et al., 2011). Earlier work provides strong evidence that when *Vibrio* biofilms are grazed by protoctistans, the bacteria release toxic compounds capable of killing the predators, the dead grazers themselves then become a meal and carbon source for the *Vibrio* (Chavez-Dozal et al., 2013). Depending on the species, and even strain type, *Vibrio* biofilms make an excellent model to determine if grazing can affect biofilm growth, structure, and production of chemicals to inhibit grazers (Barker and Brown, 1994). Current microbial selection studies are ongoing to examine adaptive responses of *V. fischeri* to various grazers and how these evolutionary outcomes impact sepiolid squid colonization. Bacteriophage, predatory bacteria (e.g., *Bdellovibrio*, *Bacteriovorax*, *Micavibrio*, and "wolfpack" feeders such as myxobacteria), and aquatic fungi also prey on the Vibrionaceae (Atlas and Bartha, 1998; Richards et al., 2012). How these natural enemies affect *V. fischeri* evolution and the sepiolid squid-*Vibrio* symbiosis are worthy of future investigations. For instance, *Vibrio* chitinases attacking fungal cell walls may be a means to avoid grazing by marine yeast. Chitinases are known to be utilized by *V. fischeri* symbionts when interacting with the squid host (Wier et al., 2010).

EVOLUTION DURING THE FREE-LIVING PHASE, ABIOTIC FACTORS, AND BACTERIAL STRESS RESPONSES

Prior work has shown that *V. fischeri* host adaptation to sepiolid squids and monocentrid fishes affects this species' ability to grow within a gradient of an abiotic factor (e.g., tolerance limits to environmental stress) while in the free-living or planktonic phase (Soto et al., 2009, 2012), implicating that natural selection could be acting on the bacterial stress responses to better accommodate the symbiont against the unprecedented stressful environments presented by a new animal host (e.g., novel immune defenses; Soto et al., 2010). The coupling of different bacterial stress responses to one another and their correlation to successful symbiosis initiation, host immunity evasion, pathogenesis, and virulence mechanisms is becoming necessary for understanding bacterial evolution (Nishiguchi et al., 2008). Future experimental evolution work will focus on the adaptability of *V. fischeri* to abiotic factor stresses, such as high and low tolerance limits of salinity, temperature, and pH while in the free-living or planktonic phase. In turn, correlated responses of *V. fischeri* adapting to these environmental stresses will be investigated in sepiolid squid hosts (Abucayon et al., 2014). Understanding how *V. fischeri* stress evolution affects its relationship with sepiolid squids will lead to new insights in the dynamic evolutionary forces that shape associations between hosts and symbionts. Because both free-living and host environments impose dramatically different selection pressures to microorganisms (e.g., evasion of immune host defenses), these perspectives have implications into infectious disease and virulence mechanisms, as genetic and physiological components responsible for mutualisms and pathogenesis are frequently identical or homologous (Ruby et al., 2005; Nishiguchi et al., 2008; Buckling et al., 2009). Stress evolution and stress-induced mutagenesis are known to be capable of creating cryptic genetic variation through varying gene-by-gene and gene-by-environment interactions which can be invisible to natural selection during the original circumstances in which they materialize but either beneficial or detrimental to bacterial fitness when conditions change (Tenaillon et al., 2004; McGuigan and Sgro, 2009; Paaby and Rockman, 2014). The evolutionary significance of cryptic genetic variation in patterning interactions between animal hosts and bacteria is unclear. *V. fischeri* adapting to a novel squid host was found to increase this symbiont's ability to form biofilms in artificial seawater containing no organic carbon while in the free-living phase. This result suggests symbiosis evolution can affect *V. fischeri*'s ability to tolerate starvation or oligotrophic conditions when subsequently outside the host (Soto et al., 2014). *V. fischeri* adapting to selective pressures imposed by abiotic factors or environmental stressors during the free-living phase may either reinforce or decouple coevolution between *Vibrio* symbionts and their animal hosts (Soto et al., 2009, 2012). A static microcosm or standing culture of *Pseudomonas fluorescens* where wrinkly spreader, fuzzy spreader, and smooth morph colonies arise over several days has become a model system for studying microbial adaptive radiation, a process known to be affected by oxygen depletion and nutrient availability (Rainey and Travisano, 1998; Travisano and Rainey, 2000). Alterations in *Vibrio* colony morphology is known to affect animal host colonization (Mandel et al., 2009). *V. fischeri* adaptive radiation during the free-living phase and the subsequent consequences on symbiosis are poorly understood. The use of microbial experimental evolution with heterogeneous environments will provide insight into how *V. fischeri* biodiversity (e.g., Shannon-Wiener Index) in the free-living phase affects symbiont population variation within the squid light organ across gradients of various abiotic factors.

METABOLISM

Biolog plates were developed for global phenotype analysis of microorganisms that allows a comprehensive survey of microbial physiological traits (Bochner, 1989; Bochner et al., 2001). The aim is to identify unique characteristics of individual microbes

and common metabolism to particular taxa or ecological populations. These plates also provide functional data to complement genetic analyses and gene expression studies of microbes. For instance, mutants can be screened efficiently to compare phenotypic consequences relative to wild type. This is especially important for examining metabolic polymorphisms, physiological heterogeneity, and distinguishing between different ecotypes within the same bacterial species, since different substrates can be shunted into alternate biochemical pathways (Rosenzweig et al., 1994; White, 2007). Additionally, how metabolism of the same substrate (i.e., D-glucose) is disproportionately distributed among numerous biochemical pathways (glycolysis versus pentose phosphate pathway) may also vary among different individual cells of the same bacterial species (Rosenzweig et al., 1994), as hypothesized by the nano-niche model of bacterial evolution (Wiedenbeck and Cohan, 2011). For example, most members of an *E. coli* population may move the carbon flow from the breakdown of D-glucose via glycolysis, but a small proportion of the remaining population may shuttle more intermediates of D-glucose degradation through Entner-Doudoroff pathway for an alternate way of making a living (e.g., physiological tradeoffs, resource partitioning, and ecological nutrient specialization by differentiation in usage of metabolic pathways; Rosenzweig et al., 1994; Cooper and Lenski, 2000; Travisano and Rainey, 2000; MacLean et al., 2004; White, 2007). Within the lifetime of just one adult squid host, a single *V. fischeri* clone has ample time to evolve cross-feeding with either other *V. fischeri* cells or host cells, since this has been documented in *E. coli* in less than 800 generations in a homogenous and unstructured environment (Rosenzweig et al., 1994). *V. fischeri* adapting to novel animal hosts undergo ecological diversification in carbon source utilization within 500 generations (Soto et al., 2014). With the use of Biolog plates, microbial experimental evolution can provide keen insight in the role of metabolism in *V. fischeri* ecological diversification and sepiolid squid colonization (MacLean and Bell, 2002).

CHEMOTAXIS

Support exists biofilms, motility, carbon metabolism, and bioluminescence are entwined or interlaced with one another. Possible crossroads for their roles in *V. fischeri* include chemotaxis, intracellular second messengers (c-di-GMP), and bacterial stress responses. Methyl-accepting chemotaxis proteins (MCPs) are central for chemotaxis, as these proteins are chemoreceptors that monitor the chemical composition of the environment and transmit this information interiorly to the cell (Bren and Eisenbach, 2000; Brennan et al., 2013). MCPs are versatile receptors to chemical stimuli, adept at mediating taxis to diverse signals (Hsing and Canale-Parola, 1996). A single MCP is incredibly sensitive. It is able to discern differences in stereochemistry between isomers, sense relative asymmetries in chemical concentrations of a substance along a gradient, and integrate diverse information of multiple chemical stimuli present in the environment simultaneously (Hsing and Canale-Parola, 1996; Bren and Eisenbach, 2000). An MCP is capable of a graded, measured, and progressive selective response to chemical stimuli. MCP function is further elaborated by being present on bacterial cell membranes as a mass complex of several interacting MCPs bundled together into a chemo-antenna cluster network, amplifying the synergistic interactions possible in chemotaxis and signal transduction (Bren and Eisenbach, 2000). Additionally, single amino acid substitutions can have colossal effects in sensitivity, affinity, specificity, and function of an MCP (Derr et al., 2006). Hence, MCPs and redistributable metabolism may allow *V. fischeri* populations to better colonize novel hosts by resculpting its N-dimensional niche hypervolume space quickly (Hutchinson, 1957). In a study using comparative genomics and a network biology-based approach to understand how genes select for multigenic phenotypes such as virulence in *V. cholerae*, loci encoding MCPs and others associated with chemotaxis were among those identified as most responsible (Gu et al., 2009). MCPs couple chemotaxis to diverse metabolites and their gradients, supplying one potential route a symbiont can adapt to unaccustomed host physiology. Experimental evolution with microorganisms to analyze chemotaxis can be completed by placing small volumes of bacteria onto the centers of motility agar plates with different chemoattractants at the periphery. Over an incubation time at an appropriate temperature, cells from the leading edge closest to the chemoattractant are serially transferred onto the centers of new motility plates (DeLoney-Marino et al., 2003). Derivations of this method can be used to select for bacteria with increased aversion to chemorepellents. Another avenue is to use a rendition of the glass capillary tube chemotaxis assay that involves continuous subculturing (Adler, 1973).

QUORUM SENSING, BIOLUMINESCENCE, SOCIAL EVOLUTION, AND ECOLOGICAL INTERACTIONS

Quorum sensing was first described in *V. fischeri* in 1970 in connection with bioluminescence (Nealson et al., 1970). Since then, quorum sensing is now known to govern many more traits other than bioluminescence, including but not limited to exoenzyme secretion, siderophore production, antibiotic synthesis, cell division, DNA replication, cell surface anabolism (cell wall, cell envelope, and capsule), biofilm development, and motility (Miller and Bassler, 2001). Bioluminescence is frequently used as a proxy quorum sensing measurement. Regulation of the *lux* operon involves input from the quorum sensing apparatus that couples to other microbial physiological pathways and cascades (Miyashiro and Ruby, 2012). Clever designs can permit microbial selection experiments that investigate quorum sensing and bioluminescence. In a plate selection scheme, ImageJ (image processing freeware produced by National Institutes of Health) may be used to single out brighter and dimmer colonies on agar plates for serial transfers that have been digitally imaged in lit and dark rooms ("digital replica plating" or "replica imaging"). The Vibrionaceae possess a hierarchical and sophisticated quorum sensing machinery comprised of "low cell density" (LCD) and "high cell density" (HCD) gene expressions (Camara et al., 2002). Microbial selection experiments with *V. fischeri* mutants locked or defaulted into LCD and HCD gene expressions will permit studies into group selection, kin selection, social evolution, and greenbeard genes (Travisano and Velicer, 2004). LCD and HCD gene expressions can each secrete a different and distinct subset of public goods not produced by the other (e.g., extracellular nuclease and metalloprotease for LCD and HCD, respectively; Blokesch and Schoolnik, 2008; Natrah et al., 2011; Bruger and Waters, 2014).

Experimentally evolved lines possessing constitutive HCD and LCD gene expressions would be compared to the quorum sensing wild type strain (ancestral or derived) for a particular selection regimen. LCD lines could serve as "cheaters" or "defectors" for a public good produced by HCD or wild type lines at high cell density (e.g., extracellular metalloproteases). An investigator could ask if an LCD cheater line initially at low frequency could invade an HCD line (at low or high cell density) or a quorum sensing wild type line at high cell density. HCD lines could analogously serve as cheaters for extracellular nuclease. The ability to control microbial growth and dilution rates with chemostats using select media might also be another way. The use of quorum sensing enhancers and quorum quenching molecules or drugs are additional avenues for future experiments (Rasmussen and Givskov, 2006; Defoirdt et al., 2008). Serial transfers of liquid cultures performed at particular cell densities (specific transmission bottlenecks) or with spent (conditioned) media may permit inquiries into quorum sensing.

Other possible ecological interactions between microbes include competition (interference and exploitation) and microbial allelopathy (e.g., chemical warfare; Atlas and Bartha, 1998). Additionally, one must recognize that one microbe may be more fit than another because of increased efficiency in resource utilization or better able to convert assimilatory carbon and reducing power into more offspring (i.e., a shorter generation time growing on D-glucose). Yet an interesting facilitation is cross-feeding. Cross-feeding can also occur between cells of different strains or species, where one cell type secretes a waste product that is utilized by another as a nutrient or useful resource. Understanding the diversity of social dynamics is valuable. Within the social evolution context, when a participant (the actor) benefits from harming another (recipient), the interaction is termed selfishness (West et al., 2006). When the actor suffers a negative effect by harming the recipient, the interaction is called spite. Altruism occurs when the recipient benefits and the actor is harmed, but mutualism takes place with both partners benefiting. Commensalism occurs if the actor benefits and the recipient experiences no effect. In amensalism, the actor is unaffected but the recipient is disserviced (Atlas and Bartha, 1998). (Predation was addressed previously.) As alluded to earlier, an initial effort to characterize the assortment of social interactions between bacteria can be done by placing washed cells in the filter-sterilized spent media of a competitor. NMR and mass spectroscopy can possibly be used to identify any interesting molecular components that can be isolated or purified. Excellent questions linger. What are the roles of cooperation, cheating, competition for limiting resources, microbial allelopathy, and other ecological interactions in shaping the squid-*Vibrio* symbiosis? At what stages do each of these processes most predominate (e.g., free-living versus host associated)? Is cheating among symbionts suppressed by the squid host when *V. fischeri* are in the light organ? Are bacteriocins produced by *V. fischeri* strains (i.e., vibriocins) against other conspecific subtypes in the squid light organ?

VIABLE BUT NON-CULTURABLE STATE

The viable but non-culturable (VBNC) state is a phenomenon frequently observed in the Vibrionaceae and other prokaryotes, including *V. fischeri* (Lee and Ruby, 1995). Bacteria normally culturable no longer grow in liquid culture or on agar media, because the cells enter a dormancy where still metabolically active and presumed to have elevated tolerance or resistance to environmental stressors (extreme conditions of an abiotic factor such as temperature or salinity), harmful compounds or noxious chemicals, starvation, and heavy metal toxicity (Ordax et al., 2006; Nowakowska and Oliver, 2013). Escape from digestion after phagocytosis or endocytosis by ameba and macrophages has also been hypothesized to be another function of the VBNC condition, permitting these eukaryotic cells to serve as reservoirs for survival and dispersal (Rahman et al., 2008). Published research has reported molecules and mechanisms (e.g., temperature upshift) that appear to restore culturability to VBNC cells upon their return to liquid media or agar plates. This putative revival of VBNC dormancy has been termed "resuscitation." However, many researchers doubt the existence of a VBNC state and its resuscitation, claiming the supporting evidence is lacking or marginal at best (Bogosian and Bourneuf, 2001). Skepticism arises because resuscitation is thought to be re-growth of injured cells that have regained their health. Disbelievers point out genes responsible for a pathway or developmental program leading to a physiologically differentiated VBNC state have been slow to identify through the use of null mutations and knockout studies (Soto et al., 2010). Nothing analogous to endospore formation has surfaced. Definitive evidence of VBNC cells will require loss-of-function experiments with subsequent complementation or overexpression gain-of-function studies to describe a "VBNC" regulon or modulon (Bogosian and Bourneuf, 2001). Microbial experimental evolution is a remarkable approach to addressing the validity of VBNC cells. After 24–48 h of growth in nutrient rich media (28°C, 200–225 rpm), most of a *V. fischeri* liquid culture is non-culturable, if not entirely dead, as the plating efficiency rapidly decreases. (Static liquid cultures do not experience this phenomenon and can remain culturable for weeks). The exact result is strain dependent, as some strains are more susceptible than others in their failure to re-grow upon subculturing to fresh media or transfer to agar plates. By serially transferring what few *V. fischeri* cells continue to grow from shaking and aging liquid cultures undergoing a decay in culturability, a population can be increasingly selected for resistance to non-culturability.

CONCLUSION

Bioinformatics will provide additional insight into experimental evolution with the Vibrionaceae, including genomics, transcriptomics, proteomics, and metabolomics. For microorganisms such as *V. fischeri*, which cycle between host-associated and free-living phases, consideration of the operating selection pressures unique to each environment, relative magnitudes, and respective contributions in driving microbial evolution merits consideration (Nyholm and Nishiguchi, 2008). Since prokaryotes possess tremendous genetic and metabolic diversity, understanding the factors that shape bacterial biogeography and ecology will provide insights into bacterial adaptation and natural history.

ACKNOWLEDGMENTS

The authors would like to thank helpful discussions with members of the Nishiguchi lab and the *Euprymna–Vibrio* community

of researchers. This work was supported by NIH NIAID 1SC1AI081659, NIH NIAIDS 3SC1AI081659-02S1, and NSF IOS 074498 to Michele K. Nishiguchi. We would also like to thank Michigan State University – BEACON Center for the Study of Evolution in Action for support.

REFERENCES

Abd, H., Saeed, A., Weintraub, A., Nair, G. B., and Sandstrom, G. (2007). Vibrio cholerae O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba *Acanthamoeba castellanii*. *FEMS Microbiol. Ecol.* 60, 33–39. doi: 10.1111/j.1574-6941.2006.00254.x

Abucayon, E., Ke, N., Cornut, R., Patelunas, A., Miller, D., Nishiguch, M. K., et al. (2014). Investigating catalase activity through hydrogen peroxide decomposition by bacterial biofilms in real time using scanning electrochemical microscopy. *Anal. Chem.* 86, 498–505. doi: 10.1021/ac402475m

Adler, J. (1973). A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *J. Gen. Microbiol.* 74, 77–91. doi: 10.1099/00221287-74-1-77

Ariyakumar, D. S., and Nishiguchi, M. K. (2009). Characterization of two host-specific genes, mannose-sensitive hemagglutinin (mshA) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. *FEMS Microbiol. Lett.* 299, 65–73. doi: 10.1111/j.1574-6968.2009.01732.x

Atlas, R. M., and Bartha, R. (1998). *Microbial Ecology: Fundamentals and Applications*. New York: Benjamin Cummings USA.

Austin, B. (2006). "Miscellaneous animal pathogens," in *The Biology of Vibrios*, eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Barker, J., and Brown, M. R. W. (1994). Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. *Microbiology*140, 1253–1159. doi: 10.1099/00221287-140-6-1253

Barrick, J. E., and Lenski, R. E. (2013). Genome dynamics during experimental evolution. *Nat. Rev. Genet.* 14, 827–839. doi: 10.1038/nrg3564

Bartlett, D. H. (2006). "Extremophilic vibrionaceae," in *The Biology of Vibrios*, eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press), 156–171. doi: 10.1128/9781555815714.ch11

Baselski, V. S., Upchurch, S., and Parker, C. D. (1978). Isolation and phenotypic characterization of virulence-deficient mutants of *Vibrio cholerae*. *Infect. Immun.* 22, 181–188.

Bennett, A. F. (2002). "Experimental evolution: an overview," in *Encyclopedia of Evolution*, ed. M. Pagel (New York: Oxford University Press), 339–342.

Bhaskaran, K., and Sinha, V. B. (1967). Attenuation of virluence in *V. cholerae*. *J. Hyg.* 65, 135–148. doi: 10.1017/S0022172400045642

Blokesch, M., and Schoolnik, G. K. (2008). The extracellular nuclease dns and its role in natural transformation of *Vibrio cholerae*. *J. Bacteriol.* 190, 7232–7240. doi: 10.1128/JB.00959-08

Bochner, B. R. (1989). Sleuthing out bacterial identities. *Nature* 339, 157–158. doi: 10.1038/339157a0

Bochner, B. R., Gadzinski, P., and Panomitros, E. (2001). Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Res.* 11, 1246–1255. doi: 10.1101/gr.186501

Bogosian, G., and Bourneuf, E. V. (2001). A matter of bacterial life and death. *Eur. Mol. Biol. Organ. Rep.* 2, 770–774. doi: 10.1093/embo-reports/kve182

Bren, A., and Eisenbach, M. (2000). How signals are heard during bacterial chemotaxis: protein-protein interactions in sensory signal propagation. *J. Bacteriol.* 182, 6865–6873. doi: 10.1128/JB.182.24.6865-6873.2000

Brennan, C. A., DeLoney-Marino, C. R., and Mandel, M. J. (2013). Chemoreceptor VfcA mediates amino acid chemotaxis in *Vibrio fischeri*. *Appl. Environ. Microbiol.* 79, 1889–1896. doi: 10.1128/AEM.03794-12

Browne-Silva, J., and Nishiguchi, M. K. (2007). Gene sequence of the pil operon reveal relationships between symbiotic strains of *Vibrio fischeri*. *Int. J. Syst. Evol. Microbiol.* 58, 1292–1299. doi: 10.1099/ijs.0.65370-0

Bruger, E., and Waters, C. M. (2014). *Quorum Sensing Inhibits the Breakdown of Cooperative Behavior by Social Cheaters in Vibrio bacteria. Evolution (N. Y.) 2014.* Raleigh: Society for the Study of Evolution.

Buckling, A., Maclean, R. C., Brockhurst, M. A., and Colegrave, N. (2009). The beagle in a bottle. *Nature* 457, 824–829. doi: 10.1038/nature07892

Camara, M., Hardman, A., Williams, P., and Milton, D. (2002). Quorum sensing in *Vibrio cholerae*. *Nat. Genet.* 32, 217–218. doi: 10.1038/ng1002-217

Cameron, D. E., and Mekalanos, J. J. (2011). "Live attenuated cholera vaccines: flagella and reactogenicity," in *Replicating Vaccines: A New Generation*, eds P. R. Dormitzer, C. W. Mandl, and R. Rappuoli (New York, NY: Springer).

Chavez-Dozal, A. A., Gorman, C., Erken, M., Steinberg, P. D., McDougald, D., and Nishiguchi, M. K. (2013). Predation response of *Vibrio fischeri* biofilms to bacterivorus protists/phagotrophic protozoa. *Appl. Environ. Microbiol.* 79, 553–558. doi: 10.1128/AEM.02710-12

Chavez-Dozal, A. A., Hogan, D., Gorman, C., Quintanal-Villalonga, A., and Nishiguchi, M. K. (2012). Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. *FEMS Microbiol. Lett.* 81, 562–573. doi: 10.1111/j.1574-6941.2012.01386.x

Chavez-Dozal, A. A., and Nishiguchi, M. K. (2011). Variation in biofilm formation among symbiotic and free-living strains of *Vibrio fischeri*. *J. Basic Microbiol.* 51, 452–458. doi: 10.1002/jobm.201000426

Colwell, R. R. (2006). "A global and historical perspective of the genus *Vibrio*," in *The Biology of Vibrios*, eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Conrad, T. M., Lewis, N. E., and Palsson, B. O. (2011). Microbial laboratory evolution in the era of genome-scale science. *Mol. Syst. Biol.* 7, 509–519. doi: 10.1038/msb.2011.42

Cooper, V. S., and Lenski, R. E. (2000). The population genetics of ecological specialization in evolving *Escherichia coli* populations. *Nature* 407, 736–739. doi: 10.1038/35037572

Criminger, J. D., Hazen, T. H., Sobecky, P. A., and Lovell, C. R. (2007). Nitrogen fixation by *Vibrio parahaemolyticus* and its implications for a new ecological niche. *Appl. Environ. Microbiol.* 73, 5959–5961. doi: 10.1128/AEM.00981-07

Crosa, J. H., Actis, L. A., and Tolmasky, M. E. (2006). "The biology and pathogenicity of *Vibrio anguillarum* and *Vibrio ordalii*," in *The Biology of Vibrios*, eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Dalia, A. B., McDonough, E., and Camilli, A. (2014). Multiplex genome editing by natural transformation. *Proc. Natl. Acad. Sci. U.S.A.* 111, 8937–8942. doi: 10.1073/pnas.1406478111

Davey, M. E., and O'toole, G. A. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* 64, 847–867. doi: 10.1128/MMBR.64.4.847-867.2000

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., and Bossier, P. (2008). Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from in vivo work. *Int. Soc. Microbial Ecol. J.* 2, 19–26. doi: 10.1038/ismej.2007.92

DeLoney-Marino, C. R., Wolfe, A. J., and Visick, K. L. (2003). Chemoattraction of *Vibrio fischeri* to serine, nucleosides, and N-acetylneuraminic acid, a component of squid light-organ mucus. *Appl. Environ. Microbiol.* 69, 7527–7530. doi: 10.1128/AEM.69.12.7527-7530.2003

Derr, P., Boder, E., and Goulian, M. (2006). Changing the specificity of a bacterial chemoreceptor. *J. Mol. Biol.* 355, 923–932. doi: 10.1016/j.jmb.2005.11.025

Dryselius, R., Kurokawa, K., and Iida, T. (2007). Vibrionaceae, a versatile bacterial family with evolutionarily conserved variability. *Res. Microbiol.* 158, 479–486. doi: 10.1016/j.resmic.2007.04.007

Dunlap, P. V. (2009). "Microbial bioluminescence," in *Encyclopedia of Microbiology*, ed. M. Schaechter (Amsterdam: Elsevier), 45–61. doi: 10.1016/B978-012373944-5.00066-3

Dunn, A. K. (2012). "*Vibrio fischeri* metabolism: symbiosis and beyond," in *Advances in Microbial Physiology: Bacterial Respiratory Physiology*, ed. R. K. Poole (London: Academic Press).

Egan, E. S., Fogel, M. A., and Waldor, M. K. (2005). Divided genomes: negotiating the cell cycle in prokaryotes with multiple chromosomes. *Mol. Microbiol.* 56, 1129–1138. doi: 10.1111/j.1365-2958.2005.04622.x

Erken, M., Weitere, M., Kjelleberg, S., and McDougald, D. (2011). *In situ* grazing resistance of *Vibrio chloerae* in the marine environment. *FEMS Microbiol. Ecol.* 76, 504–512. doi: 10.1111/j.1574-6941.2011.01067.x

Farmer III, J. J., and Janda, J. M. (2005). "Vibrionaceae," in *Bergey's Manual Systematic Bacteriology*, eds D. J. Brenner, N. R. Krieg, and J. R. Staley (New York: Springer-Verlag).

Farmer III, J. J., Janda, J. M., Brenner, D. J., Krieg, N. R., and Staley, J. R. (2005). "*Vibrio*," in *Bergey's Manual Systematic Bacteriology*, eds D. J. Brenner, N. R. Krieg, and J. R. Staley (New York: Springer-Verlag), 494–546.

Fidopiastis, P. M., von Boletzky, S., and Ruby, E. G. (1998). A new niche for Vibrio logei, the predominant light organ symbiont of squids in the genus *Sepiola*. *J. Bacteriol.* 180, 59–64.

Frey, J. (2007). Biological safety concepts of genetically modified live bacterial vaccines. *Vaccine* 25, 5598–5605. doi: 10.1016/j.vaccine.2006.11.058

Fu, X. T., and Kim, S. M. (2010). Agarase: review of major sources, categories, purification method, enzyme characteristics and applications. *Mar. Drugs* 8, 200–218. doi: 10.3390/md8010200

Gatesoupe, F. J. (1999). The use of probiotics in aquaculture. *Aquaculture* 180, 147–165. doi: 10.1016/S0044-8486(99)00187-8

Gilbert, P., Das, J., and Foley, I. (1997). Biofilms susceptibility to antimicrobials. *Adv. Dent. Res.* 11, 160–167. doi: 10.1177/08959374970110010701

Grimes, D. J., Johnson, C. N., Dillon, K. S., Flowers, A. R., Noriea , N. F. III and Berutti, T. (2009). What genomic sequence information has revealed about *Vibrio* ecology in the ocean-a review. *Microb. Ecol.* 58, 447–460. doi: 10.1007/s00248-009-9578-9

Gu, J., Wang, Y., and Lilburn, T. (2009). A comparative genomics, network-based approach to understanding virulence in *Vibrio cholerae*. *J. Bacteriol.* 191, 6262–6272. doi: 10.1128/JB.00475-09

Guerrero-Ferreira, R., Clayton Gorman, C., Chavez, A. A., Willie, S., and Nishiguchi, M. K. (2013). Characterization of the bacterial diversity in Indo-West Pacific loliginid and sepiolid squid light organs. *Microb. Ecol.* 65, 214–226. doi: 10.1007/s00248-012-0099-6

Guerrero-Ferreira, R. C., and Nishiguchi, M. K. (2007). Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis, Loliolus,* and *Euprymna* (Mollusca: Cephalopoda). *Cladistics* 23, 497–506. doi: 10.1111/j.1096-0031.2007.00155.x

Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annu. Rev. Microbiol.* 57, 249–273. doi: 10.1146/annurev.micro.57.030502.091014

Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., et al. (2000). DNA sequence of both chromosomes of the cholerae pathogen *Vibrio cholerae*. *Nature* 406, 477–483. doi: 10.1038/35020000

Hentschel, U., Steinert, M., and Hacker, J. (2000). Common molecular mechanisms of symbiosis and pathogenesis. *Trends Microbiol.* 8, 226–231. doi: 10.1016/S0966-842X(00)01758-3

Honda, T., and Finkelstein, R. A. (1979). Selection and characteristics of a *Vibrio cholerae* mutant lacking the A (ADP-ribosylating) portion of the cholera enterotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 76, 2052–2056. doi: 10.1073/pnas.76.4.2052

Hsing, W., and Canale-Parola, E. (1996). Methyl-accepting protein involved in multiple-sugar chemotaxis by *Cellulomonas gelida*. *J. Bacteriol.* 178, 5153–5158.

Hulsmann, A., Roschle, T. M., Kong, I.-S., Hassan, H. M., Beam, D. M., and Oliver, J. D. (2003). RpoS-dependent stress response and exoenzyme production in *Vibrio vulnificus*. *Appl. Environ. Microbiol.* 69, 6114–6120. doi: 10.1128/AEM.69.10.6114-6120.2003

Hutchinson, G. E. (1957). Concluding remarks. *Cold Spring Harb. Symp. Quant. Biol.* 22, 415–427. doi: 10.1101/SQB.1957.022.01.039

Jones, B. W., Lopez, J. E., Huttenburg, J., and Nishiguchi, M. K. (2006). Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol. Ecol.* 15, 4317–4329. doi: 10.1111/j.1365-294X.2006.03073.x

Jones, B. W., and Nishiguchi, M. K. (2004). Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). *Mar. Biol.* 144, 1151–1155. doi: 10.1007/s00227-003-1285-3

Kawecki, T. J., Lenski, R. E., Ebert, D., Hollis, B., Olivieri, I., and Whitlock, M. C. (2012). Experimental evolution. *Trends Ecol. Evol.* 27, 547–560. doi: 10.1016/j.tree.2012.06.001

Kolsto, A.-B. (1999). Time for a fresh look at the bacterial chromosome. *Trends Microbiol.* 7, 223–226. doi: 10.1016/S0966-842X(99)01519-X

Lee, K.-H., and Ruby, E. G. (1994a). Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. *J. Bacteriol.* 176, 1985–1991.

Lee, K.-H., and Ruby, E. G. (1994b). Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl. Environ. Microbiol.* 60, 1565–1571.

Lee, K.-H., and Ruby, E. G. (1995). Symbiotic role of the viable but nonculturable state of *Vibrio fischeri* in Hawaiian coastal seawater. *Appl. Environ. Microbiol.* 61, 278–283.

Lenski, R. E. (1995). "Evolution in experimental populations of bacteria," in *Population Genetics of Bacteria*, eds S. Baumberg, J. P. W. Young, E. M. H. Wellington, and J. R. Saunders (Cambridge: Cambridge University Press), 193–215.

Lenski, R. E. (2002). "Experimental evolution: a long-term study with *E. coli*." in *Encyclopedia of Evolution*, ed. M. Pagel (New York: Oxford University Press), 342–344.

Lenski, R. E., Rose, M. R., Simpson, S. C., and Tadler, S. C. (1991). Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am. Nat.* 138, 1315–1341. doi: 10.1086/285289

MacLean, D., Jones, J. D. G., and Studholme, D. J. (2009). Application of 'next-generation' sequencing technologies to microbial genetics. *Nat. Rev. Microbiol.* 7, 287–296. doi:10.1038/nrmicro2088

MacLean, R. C., and Bell, G. (2002). Experimental adaptive radiation in *Pseudomonas*. *Am. Nat.* 160, 569–581. doi: 10.1086/342816

MacLean, R. C., Bell, G., and Rainey, P. B. (2004). The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8072–8077. doi: 10.1073/pnas.0307195101

Madigan, M. T., and Martinko, J. M. (2006). *Brock Biology of Microorganisms*. Upper Saddle River, NJ: Pearson Pratice Hall.

Mandel, M. J., Wollenberg, M. S., Stabb, E. V., Visick, K. L., and Ruby, E. G. (2009). A single regulatory gene is sufficient to alter bacterial host range. *Nature* 458, 215–218. doi: 10.1038/nature07660

Mansson, M., Gram, L., and Larsen, T. O. (2011). Production of bioactive secondary metabolites by marine vibrionaceae. *Mar. Drugs* 9, 1440–1468. doi: 10.3390/md9091440

Matz, C., and Kjelleberg, S. (2005). Off the hook- how bacteria survive protozoan grazing. *Trends Microbiol.* 13, 302–307. doi: 10.1016/j.tim.2005.05.009

Matz, C., Moreno, A. M., Alhede, M., Manefield, M., Hauser, A. R., Givskov, M., et al.(2008). *Psuedomonas aeruginosa* uses type III secretion system to kill biofilm-associated amoebae. *Int. J. Syst. Evol. Microbiol.* 2, 843–852.

McCarter, L. L. (2001). Polar flagellar motility of the *Vibrionaceae*. *Microbiol. Mol. Biol. Rev.* 65, 445–462. doi: 10.1128/MMBR.65.3.445-462.2001

McFall-Ngai, M. J., and Ruby, E. G. (1991). Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* 254, 1491–1494. doi: 10.1126/science.1962208

McGuigan, K., and Sgro, C. M. (2009). Evolutionary consequences of cryptic genetic variation. *Trends Ecol. Evol.* 24, 305–311. doi: 10.1016/j.tree.2009.02.001

Miller, M. B., and Bassler, B. L. (2001). Quorum sensing in bacteria. *Ann. Rev. Microbiol.* 55, 165–199. doi: 10.1146/annurev.micro.55.1.165

Millikan, D., and Ruby, E. G. (2004). *Vibrio fischeri* flagellin a is essential for normal motility and for symbiotic competence during initial squid light organ colonization. *J. Bacteriol.* 185, 3547–3557. doi: 10.1128/JB.185.12.3547-3557.2003

Miyamoto, N., and Eguchi, M. (1997). Response to low osmotic stress in a fish pathogen, *Vibrio anguillarum*. *FEMS Microbiol. Lett.* 22, 225–231. doi: 10.1111/j.1574-6941.1997.tb00374.x

Miyashiro, T., and Ruby, E. G. (2012). Shedding light on bioluminescence regulation in *Vibrio fischeri*. *Mol. Microbiol.* 84, 795–806. doi: 10.1111/j.1365-2958.2012.08065.x

Moltschaniwskyj, N. A. (2004). Understanding the process of growth in cephalopods. *Mar. Fresh. Res.* 55, 379–386. doi: 10.1071/MF03147

Natrah, F. M. I., Ruwandeepika, H. A. D., Pawar, S., Karunasagar, I., Sorgeloos, P., Bossier, P., et al. (2011). Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. *Vet. Microbiol.* 154, 124–129. doi: 10.1016/j.vetmic.2011.06.024

Nealson, K. H., Platt, T., and Hastings, J. W. (1970). Cellular control of the synthesis and activity of the bacterial bioluminescent system. *J. Bacteriol.* 104, 313–322.

Ninin, E., Caroff, N., Kouri, E., Espaze, E., Richet, H. H., Quilici, M. L., et al. (2000). Nontoxigenic *Vibrio cholerae* O1 bacteremia: case report and review. *Eur. J. Clin. Microbiol. Infect. Dis.* 19, 488–491. doi: 10.1007/s100960000296

Nishiguchi, M. K. (2000). Temperature affects species distribution in symbiotic populations of *Vibrio* spp. *Appl. Environ. Microbiol.* 66, 3550–3555. doi: 10.1128/AEM.66.8.3550-3555.2000

Nishiguchi, M. K. (2002). Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microb. Ecol.* 44, 10–18. doi: 10.1007/BF03036870

Nishiguchi, M. K., DeVinney, R., Hirsch, A. M., Riley, M., Mansky, L., and Vendatum, G. (2008). Evolution of virulence: deciphering the mechanisms between pathogenic and benign symbioses. *Vie Et Milieu-LifeEnviron.* 58, 175–184.

Nishiguchi, M. K., Lopez, J. E., and Boletzky, S. V. (2004). Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. *Evol. Dev.* 6, 41–49. doi: 10.1111/j.1525-142X.2004.04009.x

Nishiguchi, M. K., and Nair, V. S. (2003). Evolution of symbiosis in the *Vibrionaceae*: a combined approach using molecules and physiology. *Int. J. Syst. Evol. Microbiol.* 53, 2019–2026. doi: 10.1099/ijs.0.02792-0

Nishiguchi, M. K., Ruby, E. G., and McFall-Ngai, M. J. (1998). Competitive dominance during colonization is an indicator of coevolution in an animal-bacterial symbiosis. *Appl. Environ. Microbiol.* 64, 3209–3213.

Nowakowska, J., and Oliver, J. D. (2013). Resistance to environmental stresses by *Vibrio* vulnificus in the viable but nonculturable state. *FEMS Microbiol. Ecol.* 84, 213–222. doi: 10.1111/1574-6941.12052

Nyholm, S. V., and Nishiguchi, M. K. (2008). The evolutionary ecology of a sepiolid squid-*Vibrio* association: from cell to environment. *Vie Et Milieu-Life Environ.* 58,175–184.

Okada, K., Tetsuya Iida, T., Kita-Tsukamoto, K., and Takeshi Honda, T. (2005). *Vibrios* commonly possess two chromosomes. *J. Bacteriol.* 187, 752–757. doi: 10.1128/JB.187.2.752-757.2005

Ordax, M., Marco-Noales, E., Lopez, M. M., and Biosca, E. G. (2006). Survival strategy of *Erwinia amylovora* against copper: induction of the viable-but-nonculturable state. *Appl. Environ. Microbiol.* 72, 3482–3488. doi: 10.1128/AEM.72.5.3482-3488.2006

Owen, J., Punt, J., and Stranford, S. (2013). *Kuby Immunology*, 7th Edn. New York, NY: W. H. Freeman and Company.

Owens, L., and Busico-Salcedo, N. (2006). "*Vibrio harveyi*: pretty problems in paradise," in *The Biology of Vibrios,* eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Paaby, A. B., and Rockman, M. V. (2014). Cryptic genetic variation: evolution's hidden substrate *Nat. Rev. Genet.* 15, 247–258. doi: 10.1038/nrg3688

Polz, M. F., Hunt, D. E., Preheim, S. P., and Weinreich, D. M. (2006). Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. *Philos. Trans. R. Soc. B* 361, 2009–2021. doi: 10.1098/rstb.2006.1928

Pruzzo, C., Vezzulli, L., and Colwell, R. R. (2008). Global impact of *Vibrio cholerae* interactions with chitin. *Environ. Microbiol.* 10, 1400–1410 doi: 10.1111/j.1462-2920.2007.01559.x

Rahman, M., Abd, H., Romling, U., Sandstrom, G., and Mollby, R. (2008). *Aeromonas–Acanthamoeba* interaction and early sh ift to a viable but nonculturable state of Aeromonas by *Acanthamoeba. J. Appl. Microbiol.* 104, 1449–1357. doi: 10.1111/j.1365-2672.2007.03687.x

Rainey, P. B., and Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature* 394, 69–72. doi: 10.1038/27900

Rasmussen, T. B., and Givskov, M. (2006). Quorum-sensing inhibitors as anti-pathogenic drugs. *Int. J. Med. Microbiol.* 296, 149–161. doi: 10.1016/j.ijmm.2006.02.005

Richards, G. P., Fay, J. P., Dickens, K. A., Parent, M. A., Soroka, D. S., Boyd, E. F., et al. (2012). Predatory bacteria as natural modulators of *Vibrio* parahaemolyticus and *Vibrio vulnificus* in seawater and oysters. *Appl. Environ. Microbiol.* 78, 7455–7466. doi: 10.1128/AEM.01594-12

Rosenzweig, R. F., Sharp, R. R., Treves, D. S., and Adams, J. (1994). Microbial evolution in a simple unstructured environment: genetic differentiation in *Escherichia coli. Genetics* 137, 903–917.

Ruby, E., Urbanowski, M., Campbell, J., Dunn, A., Faini, M., Gunsalus, R., et al. (2005). Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3004–3009. doi: 10.1073/pnas.0409900102

Sawabe, T., Sugimura, I., Ohtsuka, M., Nakano, K., Tajima, K., Ezura, Y., et al. (1998). *Vibrio halioticoli* sp. nov., a non-motile alginolytic marine bacterium isolated from the gut of the abalone Haliotis discus hannai. *Int. J. Syst. Bacteriol.* 48, 573–580. doi: 10.1099/00207713-48-2-573

Schuster, B. M., Perry, L. A., Cooper, V. S., and Whistler, C. A. (2010). Breaking the language barrier: experimental evolution of non-native *Vibrio fischeri* in squid tailors luminescence to the host. *Symbiosis* 51, 85–96. doi: 10.1007/s13199-010-0074-2

Soto, W., Gutierrez, J., Remmenga, M. D., and Nishiguchi, M. K. (2009). Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. *Microb. Ecol.* 57, 140–150. doi: 10.1007/s00248-008-9412-9

Soto, W., Lostroh, C. P., and Nishiguchi, M. K. (2010). "Physiological responses to stress in the *Vibrionaceae*," in *Cooperation and Stress in Biology: Joint Ventures in Biology*, eds J. Seckback and M. Grube (New York: Springer), 407–426.

Soto, W., Punke, E. B., and Nishiguchi, M. K. (2012). Evolutionary perspectives in a mutualism of sepiolid squid and bioluminescent bacteria: combined usage of microbial experimental evolution and temporal population genetics. *Evolution* 66, 1308–1321. doi: 10.1111/j.1558-5646.2011.01547.x

Soto, W., Rivera, F. M., and Nishiguchi, M. K. (2014). Ecological diversification of *Vibrio fischeri* serially passaged for 500 generations in novel squid host *Euprymna tasmanica. Microb. Ecol.* 67, 700–721. doi: 10.1007/s00248-013-0356-3

Sozhamannan, S., and Yildiz, F. H. (2011). "Diversity and genetic basis of polysaccharide biosynthesis in *Vibrio cholerae*,"in *Epidemiological and Molecular Aspects On Cholera*, eds S. K. Bhattacharya and T. Ramamurthy (London: Springer).

Stabb, E. V., and Visick, K. L. (2013). "*Vibrio fischeri*: squid symbiosis," in *The Prokaryotes,* eds E. Rosenberg, E. F. Delong, E. Stackebrand, S. Lory, and F. Thompson (Berlin: Springer).

Stoodley, P., Sauer, D., Davies, G., and Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Ann. Rev. Microbiol.* 56, 187–209. doi: 10.1146/annurev.micro.56.012302.160705

Tagomori, K., Iida, T., and Honda, T. (2002). Comparison of genome structures of vibrios, bacteria possessing two chromosomes. *J. Bacteriol.* 184, 4351–4358. doi: 10.1128/JB.184.16.4351-4358.2002

Tenaillon, O., Denamur, E., and Matic, I. (2004). Evolutionary significance of stress-induced mutagenesis in bacteria. *Trends Microbiol.* 12, 264–270. doi: 10.1016/j.tim.2004.04.002

Thompson, F. L., and Swings, J. (2006). "Taxonomy of the vibrios," in *Biology of the Vibrios,* eds B. A. F. L Thompson and J. Swings (Washington, DC: ASM Press), 29–43. doi: 10.1128/9781555815714.ch3

Travisano, M., and Rainey, P. B. (2000). Studies of adaptive radiation using model microbial systems. *Am. Nat.* 156, S35–S44. doi: 10.1086/303414

Travisano, M., and Velicer, G. J. (2004). Strategies of microbial cheater control. *Trends Microbiol.* 12, 72–78. doi: 10.1016/j.tim.2003.12.009

Urakawa, H., and Rivera, N. G. N. G. (2006). "Aquatic environment," in *The Biology of Vibrios,* eds F. L. Thompson, B. Austin, and J. Swings (Washington, DC: ASM Press).

Urbanczyk, H., Ast, J. C., and Dunlap, P. V. (2011). Phylogeny, genomics, and symbiosis of *Photobacterium. FEMS Microbiol. Rev.* 35, 324–342. doi: 10.1111/j.1574-6976.2010.00250.x

Ventosa, A. (2005). "Salinovibrioin," in *Bergey's Manual Systematic Bacteriology: The Proteobacteria. Springer-Verlag,* eds D. J. Brenner, N. R. Krieg, and J. R. Staley (New York: Springer-Verlag), 552–555.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., et al. (2008). Living on a surface: swarming and biofilm formation. *Trends Microbiol.* 16, 496–506. doi: 10.1016/j.tim.2008.07.004

Waldor, M. K., and RayChaudhuri, D. (2000). Treasure trove for cholera research. *Nature* 406, 469–470. doi: 10.1038/35020178

Wang, Z., O'Shaughnessy, T. J., Soto, C. M., Rahbar, A. M., Robertson, K. L., Nikolai Lebedev, N., et al. (2012). Function and regulation of *Vibrio campbellii* proteorhodopsin: acquired phototrophy in a classical organoheterotroph. *PLoS ONE* 7:e38749. doi: 10.1371/journal.pone.0038749.g001

West, S. A., Griffin, A. S., Gardner, A., and Diggle, S. P. (2006). Social evolution theory for microorganisms. *Nat. Rev. Microbiol.* 4, 597–607. doi: 10.1038/nrmicro1461

White, D. (2007). *The Physiology and Biochemistry of the Prokaryotes.* Oxford: Oxford University Press.

Wiedenbeck, J., and Cohan, F. M. (2011). Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS Microbiol. Rev.* 35, 957–976. doi: 10.1111/j.1574-6976.2011.00292.x

Wier, A. M., Nyholm, S. V., Mandel, M. J., Massengo-Tiassé, R. P., Schaefer, A. L., Koroleva, I., et al. (2010). Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2259–2264. doi: 10.1073/pnas.0909712107

Wollenberg, M. S., and Ruby, E. G. (2009). Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* from two Oahu populations. *Appl. Environ. Microbiol.* 75, 193–202. doi: 10.1128/AEM.01792-08

Wollenberg, M. S., and Ruby, E. G. (2012). Phylogeny and fitness of *Vibrio fischeri* from the light organs of *Euprymna scolopes* in two Oahu, Hawaii populations. *Int. Soc. Microbial Ecol. J.* 6, 352–362.

Wong, H. C., and Wang, P. (2004). Induction of viable but nonculturable state in *Vibrio* parahaemolyticus and its susceptibility to environmental stresses. *J. Appl. Microbiol.* 96, 359–366. doi: 10.1046/j.1365-2672.2004.02166.x

Yildiz, F. H., and Visick, K. L. (2009). Vibrio biofilms: so much the same yet so different. *Trends Microbiol.* 17, 109–118. doi: 10.1016/j.tim.2008.12.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 May 2014; accepted: 20 October 2014; published online: 09 December 2014.

*Citation: Soto W and Nishiguchi MK (2014) Microbial experimental evolution as a novel research approach in the Vibrionaceae and squid-Vibrio symbiosis. Front. Microbiol. **5**:593. doi: 10.3389/fmicb.2014.00593*

This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology.

Chapter 15
Euprymna hyllebergi and *Euprymna tasmanica*

Jaruwat Nabhitabhata and Michelle K. Nishiguchi

Abstract Bobtail squids of the genus *Euprymna* are small in size with a benthic habit. Such small size results in their insignificance in fisheries and aquaculture focused for human consumption. The unique ability of the voluntary adhesion system and symbiotic bacteria used for bioluminescence is now a primary research focus with potential industrial and biomedical applications. Their small size is well suited for the home aquarium with small volume. Culture of this cephalopod group can therefore serve both research and recreational purposes. Aquaculture in the laboratory provides valuable information for culture methodology that is utilized throughout the entire life cycle of several consecutive generations. This small size and benthic habit of *Euprymna* are advantageous for small-scale closed or open seawater culture systems. Major trends for culturing *Euprymna* are similar to other cephalopod groups, particularly benthic octopus that also produce planktonic hatchlings. Reduction of the cost of production is necessary for future large-scale production, with novel protocols for live feed requirements of planktonic young in the nursing phase.

Keywords *Euprymna* · Small size · Benthic habit · Small-scale culture · Closed and open seawater systems · Research and recreational purposes

15.1 Importance of the Species

The Thai bobtail squid, *Euprymna hyllebergi* Nateewathana 1997, is a common species occurring in the Andaman Sea of Thailand (Indian Ocean) and the Gulf of Thailand (Pacific Ocean; Nateewathana 1997; Nateewathana et al. 2001; Aungtonya et al. 2011). This species is small (20–40-mm mantle length, ML),

J. Nabhitabhata (✉)
Excellence Centre for Biodiversity of Peninsular Thailand, Faculty of Science,
Prince of Songkla University, Hatyai, Songkhla 90112, Thailand
e-mail: jaruwat.n5@gmail.com; e-mail: jaruwat.n@psu.ac.th

M. K. Nishiguchi
Department of Biology – MSC 3AF, New Mexico State University,
PO Box 30001, Las Cruces, NM 88003-8001, USA
e-mail: nish@nmsu.edu

J. Iglesias et al. (eds.), *Cephalopod Culture,* DOI 10.1007/978-94-017-8648-5_15,

Fig. 15.1 Partial sand-coated *Euprymna hyllebergi* shortly after emerging from its burrowing site. (Photograph of J Nabhitabhata)

neritic and strictly nektobenthic, inhabiting coastal waters in a similar manner to its congeners that occur in the Indo-west Pacific region (Summers 1985; Norman and Lu 1997; Reid and Jereb 2005). *E. tasmanica* (Pfeffer 1884), otherwise known as the "southern dumpling squid", is a species that resides around the continent of Australia and is found in similar habitats as *E. hyllebergi* (Reid and Jereb 2005). *E. tasmanica* is somewhat larger in size than *E. hyllebergi,* with an approximate ML of 30–40 mm (Norman and Lu 1997). In Thailand, *E. hyllebergi* and congeners are captured as by-catch of commercial fishing, particularly push netting and trawling (Nateewathana 1997). The yields are discarded as trash fishes due to their small size and low economic value. Because of this, fishing statistics of both species are not available (Nateewathana et al. 2001; Reid and Norman 1998; Reid and Jereb 2005).

Although the economic value of *Euprymna* as human food is low, there is a growing importance of the animals as scientific experimental models. The unique behaviour of *Euprymna* is its capability to retain a "coat" of sand or other debris on its dorsum (Fig. 15.1) when it emerges from its daily buried state to hunt prey at night (Anderson et al. 2002). The function of the sand coat is presumably for camouflage, making the squid difficult to be detected visually by its predators and prey (Anderson and Mather 1996; Shears 1988). The stickiness of the sand coat depends upon secretions of the ectodermal epithelium (Moynihan 1982). Choice between being sticky and nonsticky is voluntary and variable (Moynihan 1982). This indicates that the ability to use a sand coat might have evolved from the initial use of the behaviour for sand consolidation when the squid buries (Shears 1988). The ability to begin sand coating starts 5–7 days after hatching, simultaneous to their burrowing ability (Nabhitabhata et al. 2005). Additionally, bobtail squids are presently used as a model organism to identify a new generation of biomimetic adhesives and marine antifouling compounds with potential industrial value (Byern and Grunwald 2010).

Symbiotic associations between *Euprymna* and the bioluminescent bacterium *Vibrio fischeri* has been a recent focus as a model for investigating the process of bacterial colonization of host tissues and its effect on host development (Ruby 1999, Ruby and Lee 1998). *V. fischeri* and other luminous bacteria form a variety of pathogenic and cooperative associations with marine animals; more recently, they are being increasingly recognized as causes of invertebrate diseases (Guerrero-Ferreira

and Nishiguchi 2011; Guerrero-Ferreira et al. 2012). Since the process of bacterial colonization of the squid light organ begins immediately after hatching (Ruby and McFall-Ngai 1992), independent aquaculture of the squids and their luminous bacterial partners could yield valuable results for biotechnological and biomedical sciences (McFall-Ngai et al. 2012; Nyholm and Nishiguchi 2008). Because of these newly developed models for basic research, the advancement of culturing techniques for species of *Euprymna* has been especially important for monitoring fitness between generations, effects of inbreeding and, more importantly, diet and stress under laboratory conditions (Nabhitabhata et al. 2005; Sinn 2005; Sinn et al. 2008; Moltschaniwskyj and Carter 2010). Additionally, more information on their development, growth and time to reproduction can indicate whether all species have similar life-history strategies, and if this is dependent upon habitat or other abiotic factors.

15.2 State of the Art

Both *E. hyllebergi* and *E. tasmanica* are small in size and found living within benthic habitats. This is an advantage to provide culture conditions on a smaller scale with lower cost and less requirement of facilities compared to those used for pelagic and large-sized species. *Euprymna* can be cultured through several consecutive generations ensuring a supply of broodstocks. Broodstocks collected from the wild can spawn in captivity and are maintained throughout the life history of the animal. *E. hyllebergi* hatchlings are fed with wild-collected live feed for approximately 30 days, but later can be trained to accept dead feed. *E. tasmanica* hatchlings are fed small mysid shrimp two times a day for approximately 6 weeks, and then moved to a diet of ghost shrimp for the duration of their lives while in captivity. Interestingly, *E. tasmanica* adults were not trained to feed on dead material, and prefer not to eat food items that do not move. These same facilities for raising juvenile squids can be used for culture throughout the squid's entire life cycle. The daily growth rate for *E. hyllebergi* is 2.4% in length and 7.5% in weight through the culture period of 100 days. Growth rates for *E. tasmanica* were approximately 3.5% in length and 10% in weight for approximately 60 days. Growing demands for these squid for use as both biotechnological and biomimetic experimental models as well as ornamental animals for home aquaria and teaching laboratories are beneficial for aquaculture and biomedicine, e.g. Nabhitabhata et al. (2005), Moltschaniwskyj et al. (2007), Sinn and Moltschaniwskyj (2005) and Sinn et al. (2008).

15.3 Broodstocks Maintenance

Broodstocks of the Thai bobtail squid, *E. hyllebergi,* are collected live from otter board trawlers and beam trawlers, operating along the eastern part of the Gulf of Thailand, South China Sea and Pacific Ocean. Onboard, the squids are maintained in cylindrical fibreglass tanks of 50-L capacity containing 30 L of fresh seawater

with aeration and then, upon landing, are transported to the cephalopod hatchery. The broodstocks are maintained in an open system of cylindrical concrete tanks of 2 m^3 with flow-through filtered seawater (for the seawater supply system in this chapter, see also Chap. 7 "Aquaculture to Restocking"). Artificial substrates, made from pieces of longitudinal-cut polyvinyl chloride (PVC) pipe (50-mm diameter, 150-mm length), are previously placed on the tank bottom as shelters or "dens".

Broodstocks of southern dumpling squids, *E. tasmanica,* are collected by seine net in shallow waters of Botany Bay, New South Wales, Australia. Adult animals are transported to running open seawater facilities located at the Sydney Institute of Marine Sciences (SIMS) at Chowder Bay, NSW. Adults are acclimated to the conditions in the tanks (34 psu, 18 °C) and transported to New Mexico State University within 2–3 days. Transport of the squids takes approximately 36 h tank to tank in aquaria bags with less than 1 L of seawater. Animals are then acclimated to the contained recirculating artificial seawater tanks (100 L) at New Mexico State University under the same culturing conditions. Each tank is divided into eight cubic sectionals (each 0.3 m^2), which holds three to four adult individuals. Sexes are continuously kept separate, since the presence of males can stress female behaviour. The only time males are placed with females is when a planned mating is scheduled. In this manner, we can document which particular male has mated with which female (and therefore, track fecundity of each female). Males are placed in the female cubical (usually at a 1:1 or 1:2 male to female ratio) and are removed anytime between 4 and 10 h. PVC pipe cut longitudinally is placed in the female cubical and used as artificial substrates for the females to lay their eggs after they have been mated.

Squids will mate and spawn in the tanks. Mating occurs without prior pair formation for *E. hyllebergi,* and controlled conditions (noting which pairs are mated, and how many times) are maintained for *E. tasmanica*. Spawning behavioural pattern is similar for both species. The male responds to the presence of a swimming female by initially approaching and then grasping her from below in a male to female neck position. The female is pulled down to the bottom where copulation takes place. Copulation takes 7–10 min and then the pair separate. Spawning is observed at dawn, 2–3 days after mating. Prior to spawning, the female investigates substrates for attaching her eggs by swimming around, and touching the substrata with the tip of her arm cone. In the tanks, the female attaches her eggs in clusters to the inner surface of the artificial substrates (Fig. 15.2). The time period for attaching is 40–60 s for one egg. Intervals between each egg attachment lengthens as the number of eggs increases, up to 2–3 min. Spawning is intermittent and irregular and may be extended over several weeks. The total number of eggs per female is about 100–470 with an average of 200 eggs. Females can spawn up to three to four clutches in her lifetime, with the number of eggs decreasing as the female becomes older (Steer et al. 2004; Nabhitabhata et al. 2005). For *E. tasmanica,* adults reared in captivity (F1 generation) live longer (2–3 months) and produce larger and more clutches per female. Wild-caught adult *E. tasmanica* at maturity produce approximately 3 clutches while in captivity, ranging from 25 to 100 eggs per clutch (with one exceptional female, which laid approximately 500 eggs in one clutch). F1

Fig. 15.2 Egg capsules of *Euprymna hyllebergi* attached to the inner side of the artificial substratum, a piece of cut polyvinyl chloride (*PVC*) pipe. (Photograph of J Nabhitabhata)

generation *E. tasmanica* females lay up to 5 clutches/lifetime, with sizes ranging from 100 to 250 eggs per clutch. Hatching rate from the F2 clutches is approximately 99% for the first clutch, with a decrease leading up to approximately 20% (~80% hatching rate) for later clutches. F1 adults are larger and thus far have lived for 1 year in captivity (Nishiguchi, unpublished). F2 adults have similar longevities, but hatching rates for the F3 generation was somewhat lower (70–80%)

15.4 Nursing of Eggs

15.4.1 Egg Characteristics

Eggs are single, stalkless and opaque white, having a droplet shape and calcareous leather-like coating capsule (Fig. 15.3). The size of each egg is about 4 mm along its major axis, 3 mm in its minor axis and weighs about 0.02 g. About 2 h after being laid, the outer coat (or capsule) turns brown, leathery and rigid in *E. hyllebergi* (Nabhitabhata et al. 2005), whereas in *E. tasmanica* the egg is orange from wild-caught specimens. In F1 and subsequent generations of *E. tasmanica,* egg capsules are white to opaque and remain so during development. This solid protection allows the developing embryo to become a "sessile organism" during the extended period of development (Boletzky 1998). Eggs are telolecithal. Asymmetric eight-cell cleavage occurs 10 h after fertilization. Clockwise rotation of the embryo occurs from days 3 to 8, at 28 °C. Organogenesis occurs from day 4. The unique bilobed character of the external yolk sac appears after day 5 when the capsule becomes more transparent and the embryo is now visible. Chromatophores develop from day 6, and four diverticula of the internal yolk sac from day 8. The first hatching occurs at day 12 (Fig. 15.4) for *E. hyllebergi,* and day 32 for *E. tasmanica*. The embryonic phase is about 12–18 days, after approximately 14 days at 28 °C for *E. hyllebergi,* and 21–28 days at 18 °C for *E. tasmanica*. The hatching period of eggs in the same clutch takes 5 days from first to the last eggs and primarily occurs on the third day. Average hatching rate is about 94% (82–100%) for both species.

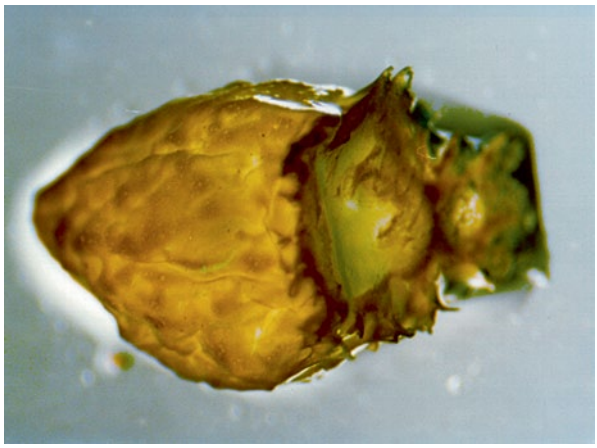


Fig. 15.3 Egg capsule of *Euprymna hyllebergi*; surface is colored brown by attached diatoms (40x). (Photograph of J Nabhitabhata)

Fig. 15.4 Hatchling of *Euprymna hyllebergi* (dorsum, 17x). (Photograph of J Nabhitabhata)

15.4.2 System Requirements and Management

Artificial substrates with egg clusters are transferred to hatch in fibreglass tanks of 50-L capacity containing 40-L filtered seawater. Two pieces of longitudinal-cut PVC pipe (25-mm diameter, 400-mm length) equipped with aeration devices are placed in each tank, facing in the same direction, to generate and direct an artificial current (Fig. 15.5). Tanks are cleaned by siphoning out the old water and replaced by a volume of 50 %. Temperature change is minimised by means of outside running water around the tank base (Nabhitabhata et al. 2005). The average temperature

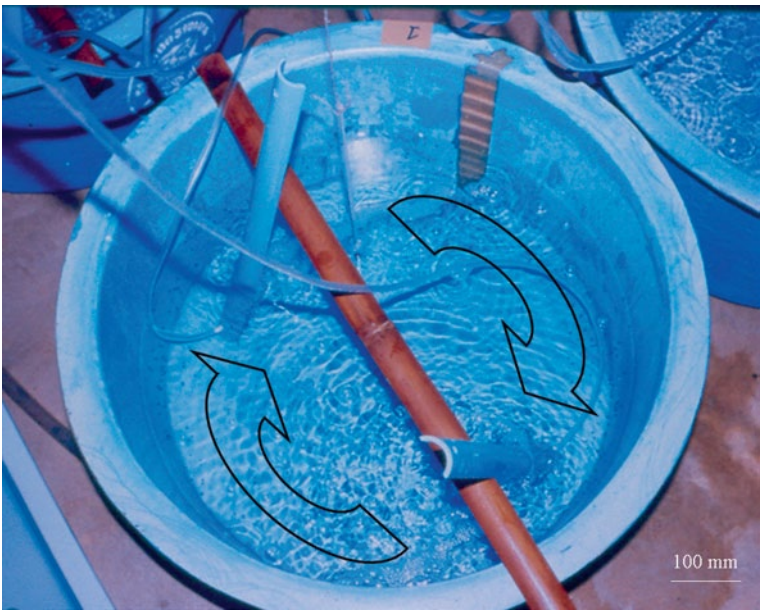


Fig. 15.5 A culture tank of *Euprymna hyllebergi* equipped with current generator devices (*arrows* indicate current direction). (Photograph of J Nabhitabhata)

can be maintained at approximately 28.2 °C, pH 8.0 and salinity at 32.5 psu. For *E. tasmanica,* clutches are placed in a 100-L polycarbonate tank with ultraviolet (UV)-filtered artificial seawater. Each individual clutch is placed in a cage made out of 2 pieces of PVC tubing, cut longitudinally and then glued back together with mesh netting in between. The clutches are placed in these cages so as not to have hatchlings mix with other clutches, as well as receiving enough aeration from below during development. Each cage is aerated with water from an individual spout that provides oxygenated seawater (Fig. 15.6). Water is kept at constant temperature (20 °C) with pH 8.0 and salinity 34.0 psu. Water changes are completed every other day to maintain salinity due to evaporation.

15.5 Nursing of Young

15.5.1 Hatchling Characteristics

The living mode of the hatchling includes a planktonic phase lasting from 6 to 8 h before the hatchling gradually adopts a benthic habit. The settling stage is approximately 5 days. Juvenile squids still enter the water column on a regular basis, alternatively planktonic and benthic, until 25–30 days after hatching. The internal yolk sac is still visible through the transparent mantle from hatching until the third day (Fig. 15.4).

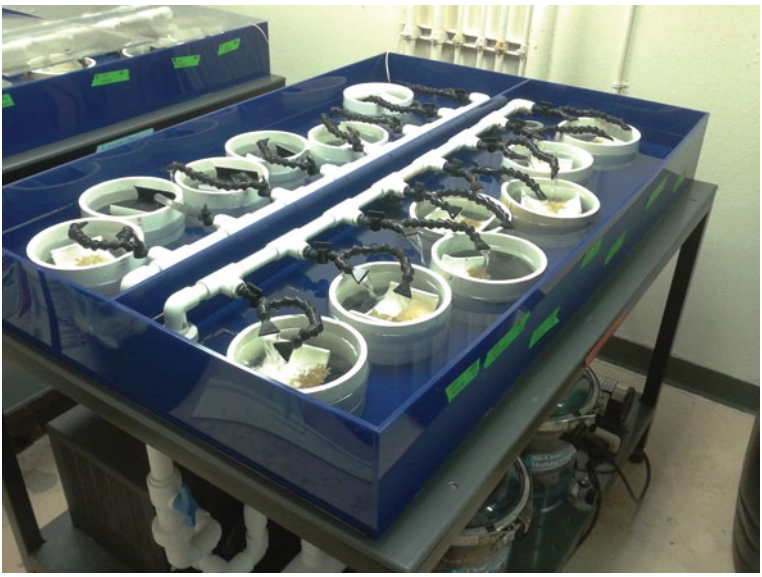


Fig. 15.6 Culture facilities for clutches/hatchlings for *Euprymna tasmanica*. (Photograph of MK Nishiguchi)

15.5.2 System Requirements and Management

Nursing of young is performed using the same system as for nursing of eggs for both species.

15.5.3 Feeding

The general task is to feed planktonic food to juvenile squids before the settling stage, at which time the squids are column feeders. Subsequently, benthic food is provided after the settling stage, when the juvenile squids settle to the bottom (Hanlon 1990; Hanlon et al. 1997; Nabhitabhata et al. 2005). *E. hyllebergi* hatchlings are fed with live, hatchery-produced penaeid shrimp larvae (*Penaeus merguiensis, P. monodon*) of the protozoea and mysis stages for 5 days after hatching (Fig. 15.7). Postlarvae of penaeid shrimps of the same species as well as wild mysids (*Mesopodopsis orientalis*) are also fed to the squids from hatching to 40 days. The planktonic young seize and eat its prey in the water column while hovering. After 25 days, the juvenile gradually changes to a benthic feeder, seizing its prey in the water column and then consuming it on the bottom substrate.

After 30 days, supplementary prey organisms for *E. hyllebergi* are palaemonid shrimps (*Palaemon styliferus*) and wild mysids (*Acetes* spp.). Training the squids to feed on dead fish meat (*Caranx leptolepis*) begins during this

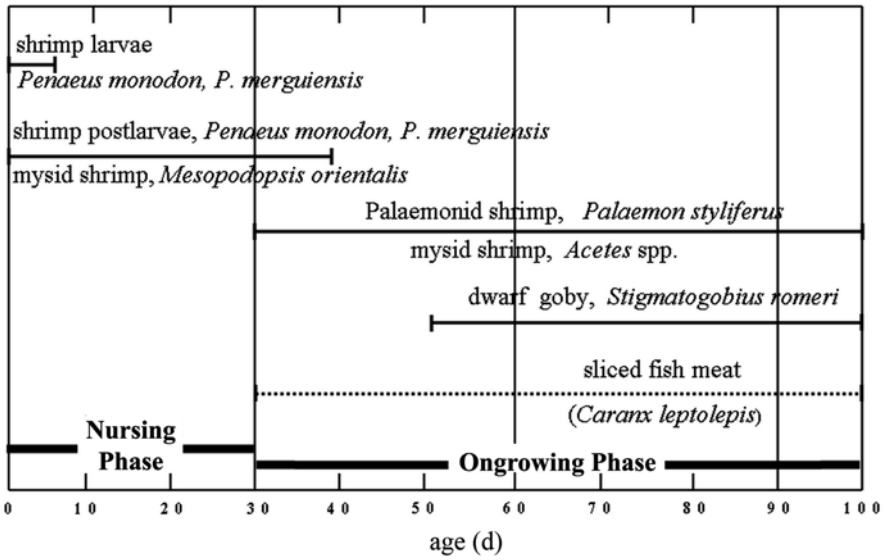


Fig. 15.7 Diagram of feeding of cultured *Euprymna hyllebergi* in the nursing phase (0–30 d) and ongrowing phase (after 30 d settlement) with live feed (*full line*) and dead feed (*dotted line*). (After Nabhitabhata et al. 2005)

period. Size grading is also initiated and continued every 10 days. The density is reduced from the initial 2–6 individuals L^{-1} by at least 25% after each grading (Nabhitabhata et al. 2005). *E. tasmanica* juveniles are fed on live, mysid shrimp for the first month (~30 days), i.e. *Tasmanomysis oculata, Paramesopodopsis refa,* and then are moved to smaller, post-larval panaeid shrimp (*Penaeus* sp.). Since *E. tasmanica* are larger when hatched, they are capable of obtaining bigger prey items earlier in their development than *E. hyllebergi*. Enriched brine shrimps (*Artemia parthenogenetica* and *A. franciscana*) can be used as substituted food when mysids are unavailable (Sinn 2005; Sinn and Moltschaniwskyj 2005; Sinn et al. 2008) although they are generally less preferred. *E. tasmanica* does not take dead prey, although there is no attempt to train juveniles to feed on this type of material.

15.5.4 Euprymna hyllebergi Growth

Hatchlings of Thai bobtail squid grow from about 2-mm ML and 0.004-g weight to 7-mm ML and 0.26 g in the first month (Fig. 15.8a, b; Nabhitabhata et al. 2005). The daily or instantaneous relative growth rate (IGR) is the highest between 10 and 20 days after hatching, about 5% in length and 17% in weight (Fig. 15.8b). The survival in the nursing phase from hatching to settling stage (0–30 days) is approximately 30%.

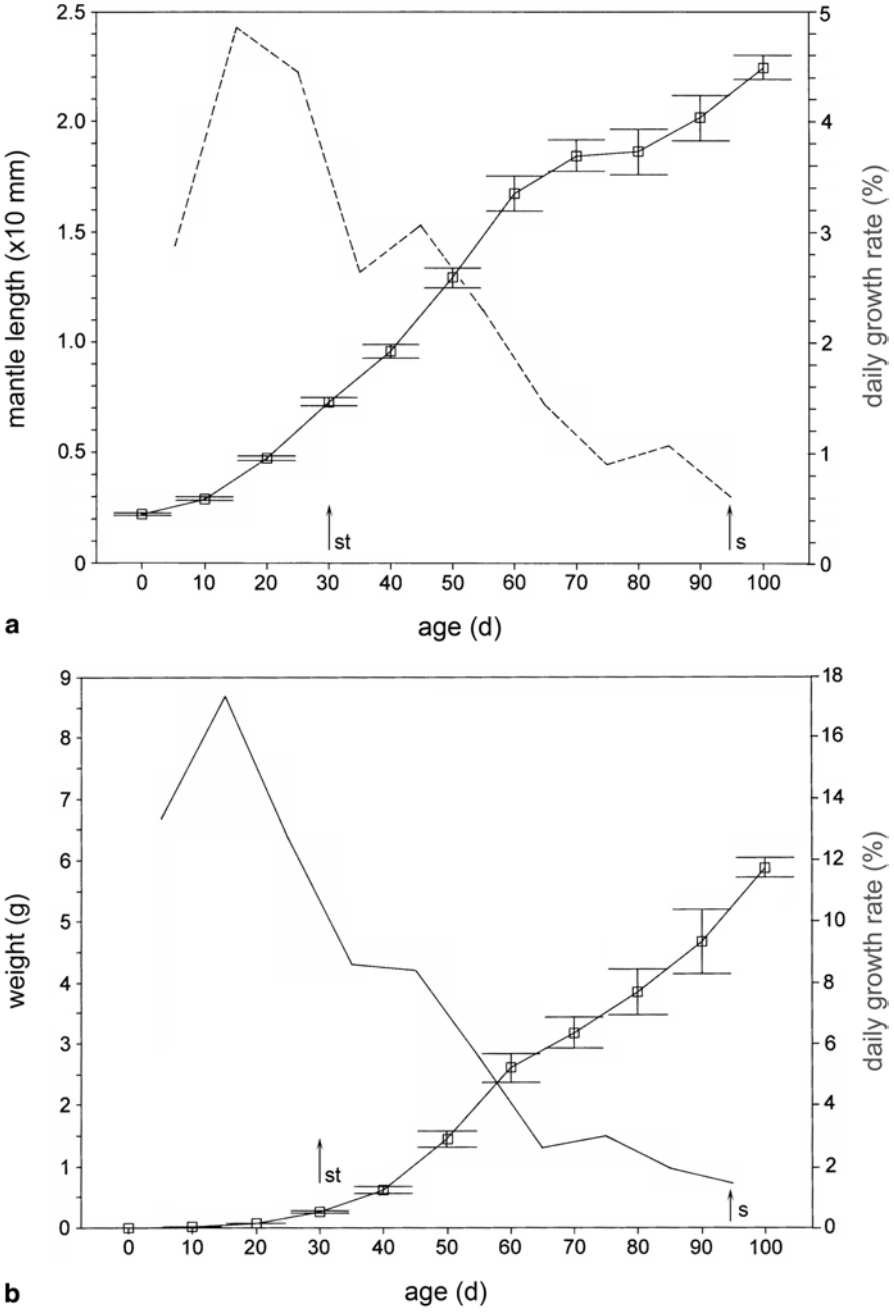


Fig. 15.8 Growth of *Euprymna hyllebergi* in terms of (*above*) mantle length (x10 mm), instantaneous relative growth rate (*IGR*: %) and age (*d*) after hatching and (*below*) weight (*g*), *IGR* (%) and age (*d*) after hatching. *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

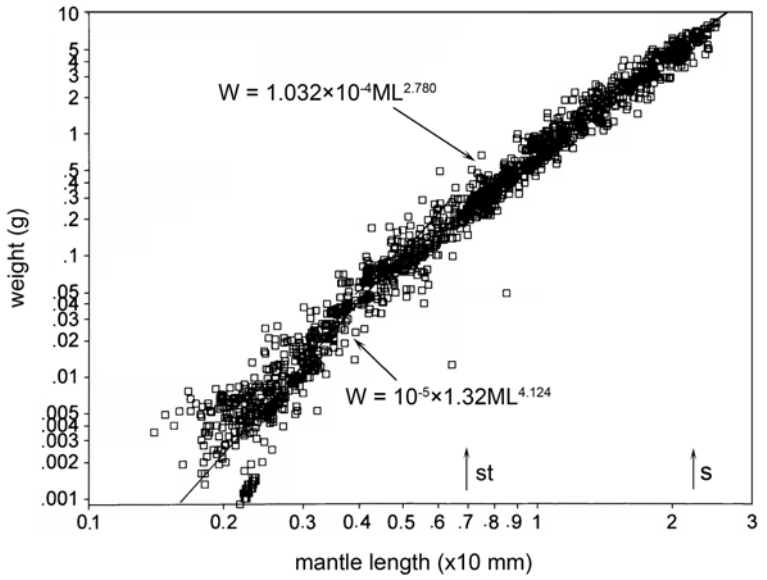


Fig. 15.9 Relationships between mantle length (x10 mm) and weight (*g*) of *Euprymna hyllebergi*; intercept of the two regressions at 5.5 mm mantle length. *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

Growth models demonstrate two phases of growth. The early phase was from hatching to 30 days where the relationships between the ML (mm) and weight (g) can be expressed as a power regression model (Fig. 15.9; Nabhitabhata et al. 2005):

$$W = 1.230 \times 10^{-4}\ ML^{4.124}. \tag{15.1}$$

The relationships between ML and age (d: days after hatching, Fig. 15.10; Nabhitabhata et al. 2005) and between weight (g) and age (d, Fig. 15.11; Nabhitabhata et al. 2005) can be expressed as the exponential models:

$$ML = 1.988e^{4.205\times10^{*}(-2)A} \tag{15.2}$$

$$W = 2.750 \times 10^{-3} e^{0.153A}. \tag{15.3}$$

15.6 Ongrowing

15.6.1 System Requirements and Management

For *E. hyllebergi,* ongrowing phase starts after the benthic young are able to accept dead fish meat. Tanks for ongrowing are the same tank used for the nursing phase and with similar management for both species. The density of *E. hyllebergi*

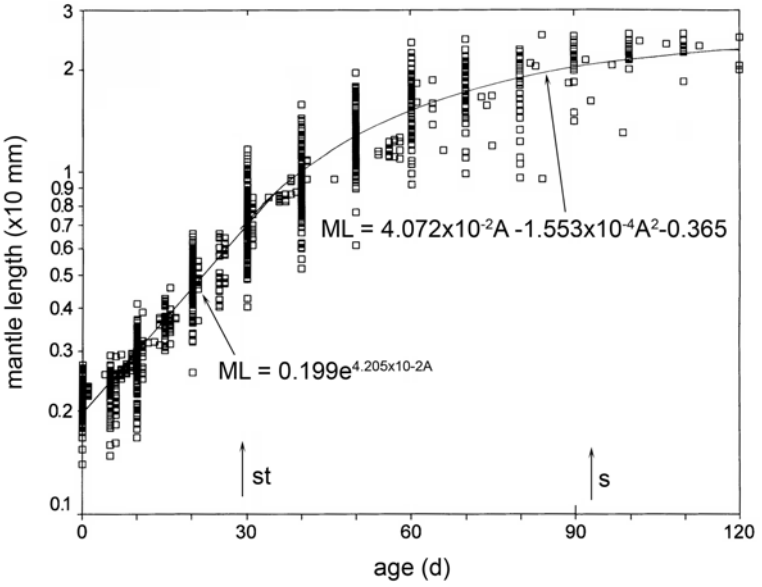


Fig. 15.10 Relationships between mantle length (x10 mm) and age (*d*) of *Euprymna hyllebergi*. *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

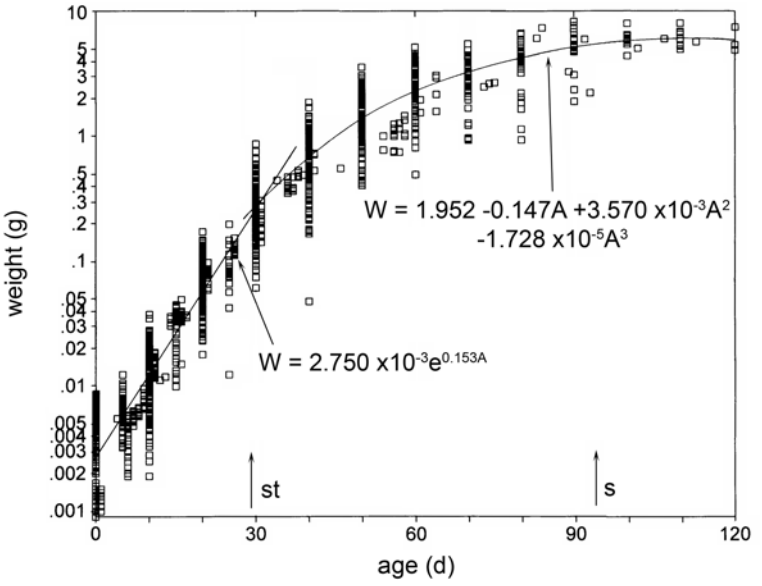


Fig. 15.11 Relationships between weight (*g*) and age (*d*) of *Euprymna hyllebergi*. *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

is changed from a water volume oriented to an (bottom) area oriented as 4–5 individuals m^{-2}. *E. tasmanica* juveniles are initially raised in round glass bowls (approximately 2 L) with sand at the bottom so the juveniles can settle. Unlike *E. hyllebergi* juveniles, *E. tasmanica* immediately settle on the bottom once they are hatched. Water is changed daily since the volume is small and there is greater evaporative loss from this volume. At approximately 2–3 weeks, juvenile squids are transferred to 40-L aquaria with sand on the bottom and raised until sexually mature (2 months). Generally, 20 squids are kept in an aquarium this size due to space limitations, but this number does not seem to affect their behaviour with any visible signs of stress. Since *E. tasmanica* F1 and F2 generations have higher growth rates than those caught in the wild, these individuals are moved earlier to the adult cubicals.

15.6.2 Euprymna hyllebergi *Growth*

The growth rate from hatching to 100 days of age for *E. hyllebergi* is approximately 2.4 % in length and 7.5 % in weight. At 60 days after hatching, the squid had grown to 17-mm length and 2.6-g weight and 22 mm and 6 g at 100 days. Food consumption of about 0.2 g d^{-1} or 37 % body weight d^{-1} enables calculation of the food conversion efficiency of about 37 % (range 14–99) from hatching to 100 days. This rate increases from 30 to 40 % after hatching to 60–70 % during 40–60 days with a peak of about 64 % between 50 and 60 days (Fig. 15.12). These values potentially relate to the storage of energy for the consequent reproductive period (Nabhitabhata et al. 2005). At 90 days after hatching, the survival from hatching is approximately 10 % and from settlement is 70 %.

Transition in growth phases is reflected in the nature of the growth models. The stage where the models shifted to a higher elevation is at about 30 days after hatching, and this corresponds to the observed settlement stage (Figs. 15.8–15.11). The second growth phase is from 30 to 122 days. The relationships between ML (mm) and weight (W, g) can also be expressed as a power regression model (Nabhitabhata et al. 2005) as happened in the early phase (Fig. 15.9):

$$W = 1.032 \times 10^{-3}\ ML^{2.780}. \tag{15.4}$$

The relationships between ML and age (d, days after hatching) and between weight (g) and age can be expressed as the quadratic equation (Fig. 15.10; Nabhitabhata et al. 2005) and a cubic regression model (Fig. 15.11; Nabhitabhata et al. 2005):

$$ML = 0.407A - 1.553 \times 10^{-3}\ A^2 - 3.648 \tag{15.5}$$

$$W = 1.952 - 0.147A + 3.570 \times 10^{-3} A^2 - 1.728 \times 10^{-5} A^3. \tag{15.6}$$

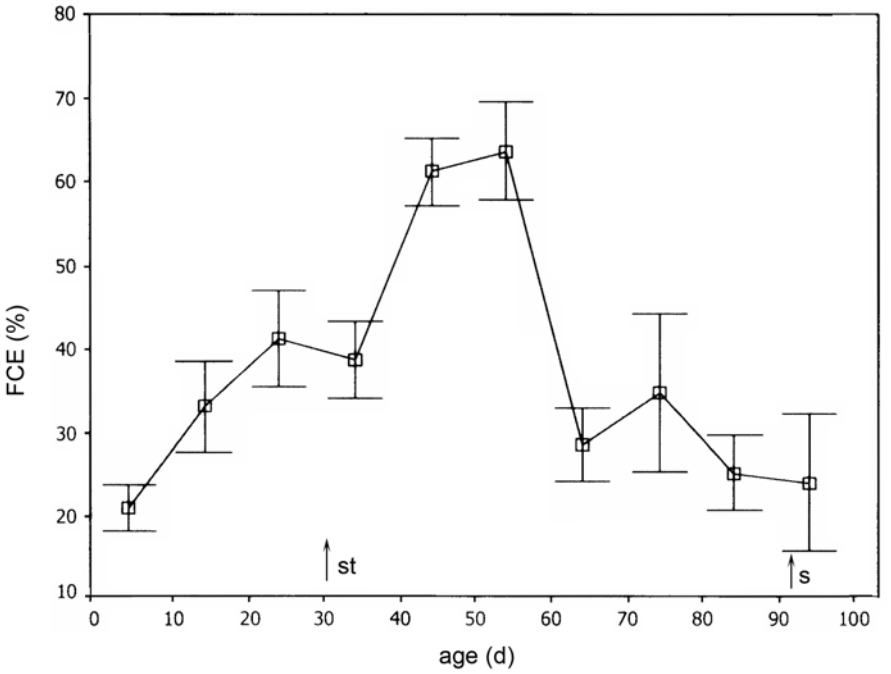


Fig. 15.12 Food conversion efficiency (*FCE*: %) of cultured *Euprymna hyllebergi* during growth (age: d). *Arrows* indicate spawning (*s*) and settling stage (*st*). (After Nabhitabhata et al. 2005)

15.6.3 Euprymna tasmanica *Growth*

Detailed studies on growth of *E. tasmanica* are scarce. Growth rate of *E. tasmanica* is rapid, with hatchlings reaching adult size in 2 months at 18 °C. *E. tasmanica* can grow from a ML of approximately 1.7 mm and a weight of 0.012 g at hatching (Steer et al. 2004) to 0.06 g at 21 days and to 6.8 g at 112 days (Sinn et al. 2008). The daily growth rate from day 21 to 63 is 7–9 %, from 63 to 84 days decreases to 2–4 % and from 84–112 days 1–2 % at 18 °C (Sinn 2005).

The relationships between weight and age are exponential from 7 to 44 days after hatching and linear from 58 to 140 days (Moltschaniwskyj and Carter 2010) which can be expressed as:

$$\mathrm{InW} = 0.069\mathrm{A} - 4.06 \tag{15.7}$$

$$\mathrm{W} = 0.07\mathrm{A} - 3.28. \tag{15.8}$$

15.7 Trends in Research and Industry

The main purpose of culture *Euprymna* is obviously not for human consumption. Present research in the fields of marine pharmacology, biotechnology and mimetic engineering requires small and “easy to culture” squids. Rapidly growing demand

in the ornamental aquaculture trade also requires organisms of similar characters, specifically size and ease of care. The small adult body size, benthic habit and good adaptability to culture conditions of *Euprymna* are prominent character suites that are well adapted to the aforementioned purposes. Based on such qualities, bobtail squids should be cultured on a small scale in order to reduce the cost of production. Additionally, a small-scale culture has advantages of the reduced size and benthic habits of the squids. The variety of flow-through open or closed seawater systems can yield different results and should be further studied to better quantify which systems are best for maximising production and those appropriate for each species.

Culture of *Euprymna* similarly encounters a bottleneck during the nursing phase similar to other cephalopods, since young innately feed on live feed. Future research should focus on developing feeds, both live and artificial. However, small-scale culture of live food organisms is more appropriate for small-scale culture of *Euprymna* in view of low operating costs at present. Development of artificial feed is necessary to reduce costs, but it could be postponed on a small scale. Artificial feed for cephalopods has not been commercially developed anywhere, but many studies are being completed, focusing on species that are aimed to be cultured as human food. Investigating various types of feed may give insight as to whether bobtail squids can also use artificial feed in such a manner.

E. hyllebergi and *E. tasmanica* can be cultured through multiple consecutive generations (3 generations for both *E. hyllebergi* and *E. tasmanica*) with similar growth rates (under similar conditions) without apparent effects of inbreeding on decreased growth (Nabhitabhata et al. 2005). Similar growth among generations enables a reliable supply of broodstocks for aquaculture and provides an alternative to continued fishing for wild-caught specimens, which can be time consuming and costly. However, the feasibility of inbreeding effects on decreasing of growth and fertility must be considered when producing future generations from the same broodstock. Broodstocks cannot rely solely on cultured batches, and wild broodstocks should be added intermittently to provide both genetic variation and possibly the induction of beneficial microbes that are necessary to keep squid healthy during their lifetime. Growth in captivity and culture methodology of both *E. hyllebergi* and *E. tasmanica* as well as their congeners should be further studied in views of maximising the aquaculture production and increasing our ability to provide a useful resource for a variety of research studies as well as the development of model aquaculture cephalopods.

15.8 Conclusions

The ability to maintain and grow small benthic squids such as *Euprymna* has opened up a new avenue for instigating the use of these animals as model systems in both bioengineering (adhesion) and biomedical (beneficial bacteria) research. The requirements for housing, maintaining and raising sepiolids is minimal and not as costly as other, more gregarious squid species, and this allows laboratories to set up facilities that may not necessarily be close to the ocean (such as NMSU). Presently, there are 14 laboratories in the USA alone that have culture facilities for raising

Euprymna; such facilities would not be feasible unless these animals were easy to transport long distances and maintained continually and without a nearby marine station or source of seawater. Additional research must be considered for the effects of inbreeding (when maintaining a constant broodstock) as well as comparing species for different traits that can be used for certain research foci. The inception of using cephalopods as research and not "feed" organisms is a new and exciting avenue that multiple areas of research can benefit from for furthering our knowledge in aquaculture, bioengineering, medicine, ecology and evolutionary biology.

References

Anderson RC, Mather JA (1996) Escape responses of *Euprymna scolopes* Berry, 1911 (Cephalopoda: Sepiolidae). J Mollusc Stud 62:543–545

Anderson RC, Mather JA, Steele CW (2002) The burying behavior of *Euprymna scolopes* Berry, 1913 (Cephalopoda: Sepiolidaae). West Soc Malacol Annu Rep 33:1–7

Aungtonya C, Nateewathana A, Tendal OS, Nabhitabhata J (2011) New records of the Bobtail Squid, *Euprymna hyllebergi* Nateewathana, 1997, with designation of a neotype. Phuket Mar Biol Cent Res Bull 70:15–27

Boletzky Sv (1998) Cephalopod eggs and egg masses. Oceanogr Mar Biol Ann Rev 36:341–371

Byern Jv, Grunwald I (eds) (2010) Biological adhesive systems from nature to technical and medical application. Springer-Verlag, Austria

Guerrero-Ferreira RC, Nishiguchi MK (2011) Bacterial biodiversity in natural environments. In: López-Pujol J (ed) Biodiversity/book 3, In Tech, Open access publisher. http://www.intechopen.com/articles/show/title/bacterial-biodiversity-in-natural-environments

Guerrero-Ferreira RC, Gorman C, Chavez AA, Willie S, Nishiguchi MK (2012) Characterization of the bacterial diversity in Indo-west Pacific loliginid and sepiolid squid light organs. Microb Ecol. doi: 10.1007/s00248–012-0099–6

Hanlon R (1990) Maintenance, rearing, and culture of teuthid and sepiolid squids. In: Gilbert DL, Adelman WJ, Arnold JM (eds) Squids as experimental animals. Plenum Press, New York

Hanlon RT, Claes MF, Ashcraft SE, Dunlap PV (1997) Laboratory culture of the sepiolid squid *Euprymna scolopes*: a model system for bacterial-animals symbiosis. Biol Bull 192:364–374

McFall-Ngai MJ, Heath-Heckman EAC, Gillette AA, Peyer SM, Harvie EA (2012) The secret languages of coevolved symbiosis: insights from the *Euprymna scolopes-Vibrio fischeri* symbiosis. Sem Immunol 24:3–8

Moltschaniwskyj NA, Carter CG (2010) Protein synthesis, degradation, and retention: mechanisms of indeterminate growth in cephalopods. Physiol Biochem Zool 83:997–1008

Moltschaniwskyj NA, Hall K, Lipinski MR, Marian JEAR, Nishiguchi MK, Sakai M, Shulman DJ, Sinclair B, Sinn DL, Staudinger M, Van Gelderen R, Villanueva R, Warnke K (2007) Ethical and welfare considerations when using cephalopods as experimental animals. Rev Fish Biol Fish 17:455–476

Moynihan M (1982) Notes on the behavior of *Euprymna scolopes* (Cephalopoda: Sepiolidae). Behav 85:25–41

Nabhitabhata J, Nilaphat P, Promboon P, Jaroongpattananon C (2005) Life cycle of cultured bobtail squid, *Euprymna hyllebergi* Nateewathana, 1997. Phuket Mar Biol Cent Res Bull 66:351–365

Nateewathana A (1997) The sepiolidae (Cephalopoda) of the Andaman Sea, Thailand, with description of *Euprymna hyllebergi* sp. nov. Phuket Mar Biol Cent Spec Publ 17:465–481

Nateewathana A, Nabhitabhata J, Nilphat P (2001) A new record of a bobtail squid, *Euprymna hyllebergi* Nateewathana, 1997, in the Gulf of Thailand. Phuket Mar Biol Cent Spec Publ 25:501–506

Norman MD, Lu CC (1997) Redescription of the southern dumpling squid *Euprymna tasmanica* and a revision of the genus *Euprymna* (Cephalopoda: Sepiolidae). J Mar Biol Assoc UK 77:1109–1137

Nyholm SV, Nishiguchi MK (2008) The evolutionary ecology of a sepiolid squid-vibrio association: from cell to environment. Vie Milieu 58:175–184

Reid AL, Jereb P (2005) Family Sepiolidae. In: Jereb P, Roper CFE (eds) Cephalopods of the world an annotated and illustrated catalogue of cephalopod species known to date vol. 1 chambered nautiluses and sepioids (Nautilidae, Sepiidae, Sepiolidae, Sepiadariidae, Idiosepiidae and Spirulidae) FAO Species catalogue for fishery purposes no. 4, vol. 1. FAO, Rome

Reid AL, Norman MD (1998) Sepiolidae bobtail squids. In: Carpenter KE, Niem VH (eds) The living marine resources of the western central Pacific vol 2 cephalopods, crustaceans, holothurians and sharks. FAO, Rome

Ruby EG (1999) The *Euprymna scolopes*-*Vibrio fischeri* symbiosis: a biomedical model for the study of bacterial colonization of animal tissue. J Molec Microbiol Biotech 1:13–21

Ruby EG, Lee K (1998) The *Vibrio fischeri*-*Euprymna scolopes* light organ association: current ecological paradigms. Appl Env Microbiol 64:805–812

Ruby EG, McFall-Ngai MJ (1992) A squid that glows in the night: development of an animal-bacterial mutualism. J Bacteriol 174:4865–4870

Shears J (1988) The use of a sand-coat in relation to feeding and diel activity in the sepiolid squid *Euprymna scolopes*. Malacologia 29:121–133

Sinn DL (2005) From individuals to populations: personality traits in southern dumpling squid (*Euprymna tasmanica* Pfeffer, 1884) and their life history correlates. Doctor of Philosophy Thesis, University of Tasmania

Sinn DL, Moltschaniwskyj NA (2005) Personality traits in dumpling squid (*Euprymna tasmanica*): context-specific traits and their correlation with biological characteristics. J Comp Psychol 119:99–110

Sinn DL, Gosling SD, Moltschaniwskyj NA (2008) Development of shy/bold behaviour in squid: context-specific phenotypes associated with developmental plasticity. Anim Behav 75:433–442

Steer MA, Moltschaniwskyj NA, Nichols DS, Miller M (2004) The role of temperature and maternal ration in embryo survival: using the dumpling squid *Euprymna tasmanica* as a model. J Exp Mar Biol Ecol 307:73–89

Summers WC (1985) Comparative life history adaptations of some myopsid and sepiolid squids. NAFO Sci Stud 9:139–142

Chavez-Dozal et al. BMC Microbiology (2015) 15:226
DOI 10.1186/s12866-015-0560-z

BMC Microbiology

RESEARCH ARTICLE Open Access

Proteomic and metabolomic profiles demonstrate variation among free-living and symbiotic *vibrio fischeri* biofilms

Alba Chavez-Dozal, Clayton Gorman and Michele K. Nishiguchi*

Abstract

Background: A number of bacterial species are capable of growing in various life history modes that enable their survival and persistence in both planktonic free-living stages as well as in biofilm communities. Mechanisms contributing to either planktonic cell or biofilm persistence and survival can be carefully delineated using multiple differential techniques (*e.g.*, genomics and transcriptomics). In this study, we present both proteomic and metabolomic analyses of *Vibrio fischeri* biofilms, demonstrating the potential for combined differential studies for elucidating life-history switches important for establishing the mutualism through biofilm formation and host colonization.

Methods: The study used a metabolomics/proteomics or "meta-proteomics" approach, referring to the combined protein and metabolic data analysis that bridges the gap between phenotypic changes (planktonic cell to biofilm formation) with genotypic changes (reflected in protein/metabolic profiles). Our methods used protein shotgun construction, followed by liquid chromatography coupled with mass spectrometry (LC-MS) detection and quantification for both free-living and biofilm forming *V. fischeri*.

Results: We present a time-resolved picture of approximately 100 proteins (2D-PAGE and shotgun proteomics) and 200 metabolites that are present during the transition from planktonic growth to community biofilm formation. Proteins involved in stress response, DNA repair damage, and transport appeared to be highly expressed during the biofilm state. In addition, metabolites detected in biofilms correspond to components of the exopolysaccharide (EPS) matrix (sugars and glycerol-derived). Alterations in metabolic enzymes were paralleled by more pronounced changes in concentration of intermediates from the glycolysis pathway as well as several amino acids.

Conclusions: This combined analysis of both types of information (proteins, metabolites) has provided a more complete picture of the biochemical processes of biofilm formation and what determines the switch between the two life history strategies. The reported findings have broad implications for *Vibrio* biofilm ecology, and mechanisms for successful survival in the host and environment.

Keywords: *V. fischeri*, Symbiosis, Biofilms, Planktonic, Mass spectrometry, Liquid chromatography, Metabolomics, Proteomics

* Correspondence: nish@nmsu.edu
Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA

Background

Among biofilm communities, there are multiple biochemical interactions that shape the dynamic community that contrasts from free-living planktonic cells. Specifically, the notable stress resistance of biofilms has been associated with physiological changes that bacteria undergo during the transition to the biofilm state [1]. A vast amount of research in the last decade has focused on characterizing unique aspects of microbial biofilms, which include genomics and post-genomic functional approaches. These techniques have allowed a comparative molecular characterization of bacterial communities during various life history stages [2–4]. Numerous techniques include 16S rRNA sequencing for community composition analysis [5], mutational analysis of particular genes, RNA profiling [6], genomics [7], transcriptomics for capturing global views of genetic diversity and expression, and isotope-probing to link phylogeny with community function and processes [8]. These pioneering studies were helpful for their initial differential analysis of biofilm life history strategies, and have opened the way to genetic characterization of biofilm-single cell transitions in phenotype.

New approaches have become available that allow a complete differential profile including proteomic and metabolomic analysis [9]. Proteomic profiles help dissect the complexity of microbial communities by analyzing protein expression, function, modification, and interactions over temporal scales. Specific separation techniques coupled with mass spectrometry (MS) analysis are also essential for proteomic profiling of these extensive and diverse populations. One classic approach is the combination of two-dimensional (2D) protein gel electrophoresis followed by spot identification via isoelectric focusing [10]. In addition, high-throughput approaches are available for protein profiling such as shotgun proteomics, where proteins are digested and the generated peptides are identified by capillary liquid chromatography in tandem with mass spectrometry [10].

Metabolomics refers to the analytical approach used to study different cell products ("chemical fingerprints") that help to understand the physiological state of microorganisms [11]. This analysis is achieved with the use of liquid chromatography coupled with mass spectrometry (LC-MS), followed by detection, and quantification. Subsequent identification of metabolites is then accomplished by cluster analysis and mapping [11]. Although each type of analysis produces an extensive amount of information, combining complementary techniques could significantly contribute to our understanding of biofilm developmental processes. A more recent approach termed "meta-proteomics" aims to identify and combine protein and metabolic data to bridge the gap between phenotypic changes (planktonic cell to biofilm formation) with genotypic changes (reflected in protein/metabolic profiles) [12]. Earlier studies have integrated various system biology analyses (including proteomics, metabolomics and transcriptomics) for biofilms formed by *Bordetella pertussis* [13], *Leptospirillum sp.* [14], and *Pseudomonas fluorescens* [15]. However, there are no studies that integrate data collected via proteomic/metabolomic (meta-proteomics) data for *Vibrio* biofilms.

Vibrio fischeri is a mutualistic bioluminescent bacterium that infects the light organs of sepiolid squids and monocentrid fishes. *V. fischeri* produces bioluminescence that is used by the squid to avoid predation in a behavior known as counterillumination [16]. The mutualism is established when the host provides an appropriate niche for the bacteria to reproduce at much higher rates than in their free-living state [17]. *V. fischeri* is capable of forming biofilms both in seawater during its free-living stage and inside its host squid's light organ while in symbiosis [18]. Environmental and mutualistic biofilms differ in the sense of bacterial diversity, where multispecies biofilms dominate the seawater environment [1] and only one or a few *Vibrio* bacteria colonize and form biofilm inside the squid's light organ [19–22]. The ability of *V. fischeri* to form a biofilm community within its squid host plays a central role in the establishment and maintenance of the mutualism, as well as the degree to what functional molecules are produced and overexpressed for biofilm formation. Therefore, this study aims to describe the metabolic and proteomic profiles of a monospecies biofilm that is crucial for understanding what bacterial molecular components are important for establishing this mutualistic association. Proteomic and metabolomic analyses for both free-living (planktonic) and monospecies biofilm were completed for *V. fischeri* strain ETJB1H to provide the first partial *Vibrio* meta-proteome profile for single cell-to-biofilm physiology. Metabolomic analysis indicates several molecular changes that are the result of different biosynthetic pathways associated with exopolyssacharide (EPS) production and biofilm formation, as well as proteins that are important for persistence in the seawater environment.

Methods

Microorganism and biofilm formation experiments

Vibrio fischeri ETJB1H was isolated from the light organ of *Euprymna tasmanica* from Jervis Bay, Australia [23] and was used throughout this study. *V. fischeri* ETJB1H was routinely cultured on Luria Broth High Salt (LBS, 10 g tryptone, 5 g yeast extract, 20 g sodium chloride, 50 mL 1 M Tris pH 7.5, 3.75 mL 80 % glycerol and 950 mL distilled water) agar (15 %) and sub-cultured on LBS liquid media at 28 °C. Biofilm formation was grown as previously described [24, 25]. Briefly, three flasks of

250 mL with 100 mL of LBS media were inoculated with a 1:100 dilution from an overnight culture (of a 1.0 McFarland standard) and incubated for 24 h at 28 °C under static conditions. After incubation, planktonic cells were removed and flasks were briefly washed with LBS to remove any excess planktonic cells. Biofilm-forming bacteria (the ones that were tightly attached to the glass of the flask) were then removed by placing 100 mL of LBS and sonicating for 10 s three times under low intensity (40 %) power using the Bransonic 220 sonicator (Branson. Ultrasonic, Danbury, CT, USA). Biofilm cells were then concentrated and washed three times by spinning down the cultures at 10,000 xg for 15 min and removing any excess supernatant media. Each of the samples were divided in equal amounts for their proteomic 2-D page and shotgun analysis respectively (3 combined samples for the 2D page analysis and 3 combined samples for the shotgun analysis).

Protein preparation and 2-D PAGE electrophoresis

For protein extraction, we used the EasyLyse™ bacterial protein extraction solution (Epicentre technologies, Madison, WI) following manufacturer's instructions. In brief, a lysis solution was prepared as follows: 0.5 mL of D.I. water, 2 μl of 1 M $MgCl_2$, and 0.5 mL of Lysis Buffer and 1 μl of enzyme were added. A cell pellet consisting of aproximatelly 10^9 cells was added to 200 μL of the above solution. After incubation at room tempareture for 5 min, the samples were centrifugated and the supernatant (cell paste) was transferred to a clean tube. Fifty micrograms of each cell paste was prepared for first dimensional isoelectric focusing by adding four parts of lysis solution (7 M urea, 2 M thiourea, 1 % dithiothreitol, 2 % Pharmalyte 3–10, 0.5 % Triton X-100, 0.14 % phenylmethylsulfonyl fluoride) to one part of protein sample (volume per volume) as described previously [26].

The proteins are initially separated in the first dimension based on their isoelectric points; the focused proteins of the first dimension are subsequentelly separated in a second dimension based on their molecular masses. First, dimensional separation was completed using 17 cm IPG strips, pH 3–10. One microgram of the protein sample was loaded and isoelectric focusing was performed following the manufacturer's protocol (Bio-Rad, Richmond, CA). PROTEAN© IEF cell was used for the first separation at settings of 150 kVh and 23 °C. Strips were then equilibrated for 15 min in a buffer containing 2 % SDS, 6 M urea, 0.05 M Tris–HCl, pH 8.5, and 20 % glycerol with 2 % DTT (Dithiothreitol) and equilibrated again in the same buffer with 2.5 % iodoacetamide. The equilibrated strips were transferred to a PROTEAN II© version xi cell tank for second dimension run (30 mA per gel) in 10 % polyacrylamide gel, and then visualized after staining with Coomasie brilliant blue R250. Stained cells were covered with cellophane and air-dried overnight at room temperature. Gels were analyzed pairwise by eye for differences in their protein patterns [26]. In addition, differential analysis by Guild BioSciences proteome analysis service and a computer densitometric analysis of spots were completed using the Image Master Platinum 5.0 software (GE Healthcare, PA). A threshold of 2-fold change was used to determine significance between biofilm and planktonic groups. Gels were analyzed pairwise by eye for differences in their protein patterns by overlaying the gels on a light table, gels were then scanned into a computer graphics program (Adobe Photoshop 5.0) and one replicated is used as a reference.

Protein shotgun analysis

A fraction of the whole protein extractions (approximately 200 μL, which corresponds to 1 mg of total protein) were trypsin-digested. For digestion, the sample was reduced by adding 5 μL of DDT (Dithiothreitol, 200 mM: 1 mL of 100 M NH_4HCO_3 and 30.86 mg of DTT) and boiled for 10 min and then incubated for 1 h. Alkylation was achieved by adding 4 μL of iodoacetamide (1 M: 200 μL of 100 mM NH_4HCO_3 and 37 mg of iodoacetamide) and incubated for 1 h. Neutralization of the remaining iodoacetamide was achieved by adding 20 μL of DTT and incubating for 1 h. Trypsin was added to the mixture (1 mg for every 50 mg of protein) and complete digestion was accomplished after incubating for 18 h at 37 °C. Protein digests (approximately 100 μM) were analyzed by tandem mass spectrometry through cation exchange-reversed phase chromatography, utilizing a hybrid linear ion trap FT-ICR mass spectrometer with ultra performance liquid chromatograph (UPLC/MS, Agilent Technologies 110 Series, CA) with a capillary system attached to a quadruple ion time (Thermo LQT, Thermo Fisher Scientific, CA). Three technical replicates were analyzed for each combined sample. Peptide libraries were collected in a database (as a single .mgf file for each sample) searched against a marged database composed of reviewed entries of Uniprot database and analyzed with Mascot search engine (www.matrixscience.com). Mascot parameters include proteolysis by trypsin/chymotrypsin with size tolerances of 0.5 Da for peptide fragments, with a 95 % probability that the protein identified is not a random match. The false discovery rate (FDR) was calculated using the automated decoy database tool in MASCOT where decoy statistics were automatically calculated for all matches. Alternatively, FASTA sequences of target peptides previously identified were run in the program peptide cutter (web.expasy.org/peptide_cutter) and resulting fragments were compared to those identified in our analysis. A score of 35 % matching peptides (or higher) indicates a protein match [27].

Metabolomic analysis

To prepare samples for metabolite extraction, strains were inoculated in triplicate in 120 mL of Luria Bertani high salt media (LBS; per litre composition: 10 g tryptone, 5 g yeast extract, 20 g NaCl, 50 ml 1 M tris pH 7.5, 3.75 ml 80 % glycerol and 950 mL dH_2O). When cultures reached an OD_{580} of 1.0, they were pelleted at 10,000 xg for 10 min at 4 °C. Pellets were re-suspended in 10 mL of ice-cold phosphate buffered saline (PBS, pH 7.4) and cells were pelleted again under the same conditions. The supernatant was discarded and pellets were snap-frozen with liquid nitrogen. Bacterial cells were lyophilized for 24 h (Labconco model 7740020) and were analyzed by the Biotechnology Center at the University of Illinois at Urbana-Champaign (Metabolomics Center, Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign). The approach used was a two-step LC/MS (Applied Biosystems 5500 QTrap with Agilent 1200 LC, Agilent Technologies, CA and Applied Biosystems CA) followed by targeted identification of differentially expressed metabolites using quadrupole time of flight (Q-TOF) MS/MS. Three technical replicates were used for this analysis.

Results

V. fischeri cells were grown in parallel as the planktonic (free-swimming) culture and as biofilms on the glass surface of the flask to identify and compare differences in protein profiles from the two physiological states. Cell viability was not affected after sonication for the biofilm cells, and there was no statistical difference between the number of Colony Forming Units of planktonic and biofilm samples collected after incubation time (24 h at 28 °C) for an OD_{600} = 1.0 (data not shown). The profiles presented correspond to a mature biofilm (structure achieved between 18 and 24 h of incubation) and free-living cells that did not form any biofilm. Two proteomic analysis approaches were used including i) Complete protein profile obtained by spot analysis, followed by differential two-dimensional gel electrophoresis, and ii) trypsin protein fractionation followed by shotgun identification (UPLC/MS) and peptide analysis (Mascot, peptide cutter). Metabolomic analysis was achieved through liquid chromatography coupled with mass spectrometry (LC/MS).

Identification of biofilm protein fractions by two-dimensional gel electrophoresis

Patterns of protein expression in biofilm communities were complex with an average of approximately 300 spots per gel. Using the planktonic protein gel as the reference, protein spots detected in the biofilm profile were matched against the reference. The number of matched spots was 140 with a total of 75 % gel coverage. Of these, 21 spots were up-regulated in a magnitude of 2 fold or more (Fig. 1 and Additional file 1: Figure S1). Table 1 lists the assay parameters used (isoelectric point and molecular weight of proteins) that correspond to spots numbered in Fig. 1. In addition, there were 59 spots that were unique to the biofilm state (not present in the planktonic stage) that are indicated in Fig. 2 and Table 2. All the proteins listed from this analysis could be detected reproducibly in the range of 15 to 130 KDa.

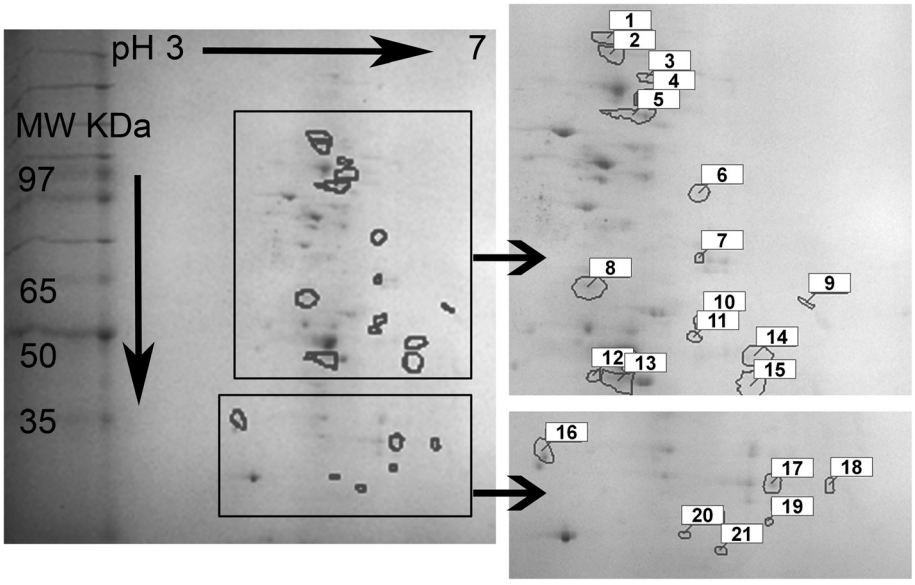


Fig. 1 2D-PAGE gel of the up-regulated *Vibrio fischeri* ETJB1H biofilm protein exudate. Colored squares correspond to magnification of fractions of the gel indicating a better resolution of spots. Numbers identify up-regulated proteins described in Table 1

Table 1 Biochemical properties of proteins identified to be up-regulated in the biofilm state of *Vibrio fischeri* ETJB1H

Spot	Isolectric point	Molecular weight (KDa)
1	3.97	111.51
2	3.98	105.53
3	4.20	96.30
4	4.22	89.63
5	4.12	84.35
6	4.52	70.82
7	4.52	59.62
8	3.85	55.37
9	5.17	53.52
10	4.53	51.82
11	4.49	50.35
12	3.88	47.64
13	4.03	47.27
14	4.86	49.00
15	4.83	46.98
16	3.22	39.60
17	4.68	35.06
18	5.05	34.74
19	4.65	29.76
20	4.12	28.51
21	4.35	30.66

Spot numbers correspond to proteins labeled in Fig. 2

Tandem mass spectrometry of peptides

The first scale proteomic analysis for *V. fischeri* using two-dimensional electrophoresis provides us with differentially expressed protein profiles that include both isoelectric points and molecular weight. In order to identify proteins that were present in the biofilm state of *V. fischeri*, a shot-gun approach was used to detect differentially expressed proteins by matching peptide mass data to available proteome sequence databases (www.ncbi.nlm.nih.gov and www.uniprot.org) using the keyword *"Vibrio fischeri* ES114". Additional analyses utilized the Mascot database. In order for a protein to be identified and considered present, tryptic peptides were required to be the primary identified hit in the database, and digests had to match at least 35 % of the complete protein compared with NCBI and Uniprot databases. In addition, theoretical isolectric points for the protein matches were calculated using algorithms from AnTheProt (antheprot-pbil.ibcp.fr) and Scansite3 (scansite3.mit.edu/#home). The criteria applied for identification resulted in a list of peptides that correlate with the molecular weight and isoelectric point detected for some protein spots observed to be either unique or up-regulated during biofilm production (Table 3).

Proteins related to multiple cellular processes were identified and listed in Table 3. The highest match corresponds to the catalytic enzyme UDP-N acetylglucosomine 1 carboxyvinyltransferase (UDP-GlcNAc) with a calculated MW/IP match to spot 33 (Table 2). Another enzyme detected corresponds to the adenosyltransferase cob(I)yrinic acid a,c-diamide transferase (MW/IP match

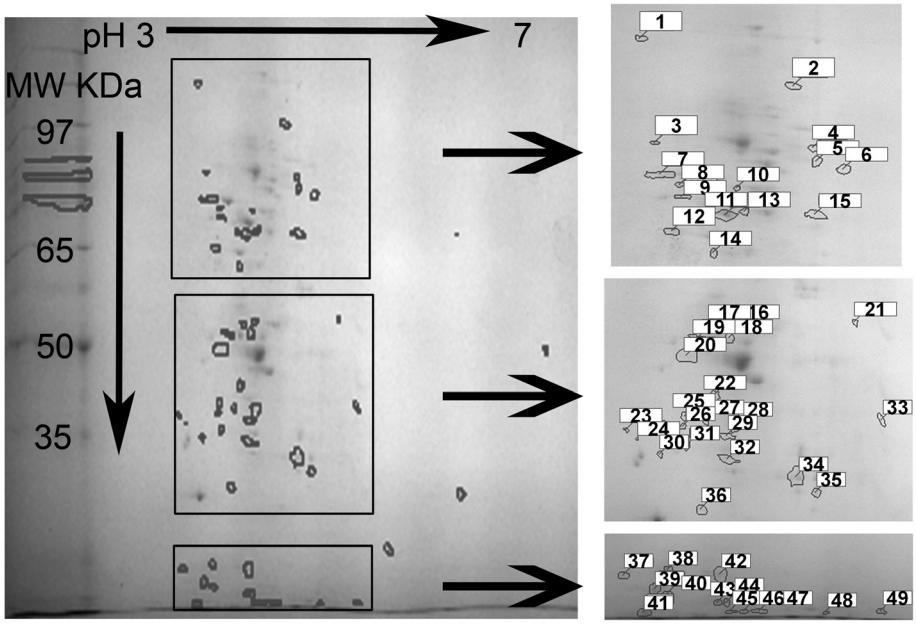


Fig. 2 2D-PAGE gel of unique *Vibrio fischeri* ETJB1H biofilm protein exudates. Colored squares correspond to magnification of fractions of the gel indicating an increased resolution of spots. Numbers identify unique biofilm proteins described on Table 2

Table 2 Biochemical properties of proteins identified to be unique in the biofilm state of *Vibrio fischeri* ETJB1H

Spot	Isolectric point	Molecular weight (KDa)
1	3.33	164.33
2	4.37	121.17
3	3.43	92.69
4	4.51	90.38
5	4.53	85.01
6	4.72	82.48
7	3.47	80.78
8	3.60	78.15
9	3.62	75.72
10	3.99	77.34
11	3.91	72.59
12	3.55	70.00
13	4.04	73.30
14	3.83	65.34
15	4.52	72.74
16	4.01	53.04
17	3.87	52.69
18	3.96	51.41
19	3.7	51.41
20	3.61	49.57
21	5.00	53.39
22	3.84	46.03
23	3.10	42.68
24	3.19	41.60
25	3.59	44.19
26	3.58	43.08
27	3.76	43.74
28	4.02	43.37
29	3.94	41.94
30	3.38	39.32
31	3.61	40.52
32	3.95	38.46
33	5.22	43.83
34	4.52	35.37
35	4.68	32.56
36	3.73	29.77
37	3.16	33.45
38	3.52	32.81
39	3.40	31.40
40	3.52	28.54
41	3.32	15.39
42	3.93	33.38
43	3.91	20.75
44	3.98	20.75
45	4.02	15.77
46	4.13	15.77
47	4.25	15.77
48	4.78	15.39
49	5.22	15.77

in #20 of Table 1). Porins and membrane transporters were detected in *V. fischeri* ETJB1H biofilms, including outer membrane protein U (OmpU; #37 in Table 2), membrane transporter ABC (#11 in Table 2), and multi-drug efflux pump (#2 in Table 2).

Interestingly, multiple stress-related proteins were detected in our study. These include the heat-stress response-related ATPase Clp protease (#6, Table 2), the carbon starvation protein A (#9, Table 1), the specific helicase RuvA related to Holiday junction formation (# 19, Table 1), the DNA double-strand repair protein RecA (#19, Table 1), the chaperone DnaK (#6, Table 1) and the transcriptional activator sigma 54 (#9, Table 1). Another set of proteins include flagellin (#13, Table 1), bioluminescence regulator (Lux R; #5, Table 1), oxidoreductase (#33, Table 1) and phosphate binding protein (#19, Table 1).

Metabolomic analysis of *Vibrio fischeri* ETJB1H biofilms

A mass spectrometry-based profiling method was used for constructing the metabolome of *V. fischeri* ETJB1H planktonic and biofilm stages. This comparison is the first metabolomic study to determine the chemical fingerprint of *V. fischeri* biofilms, as well as the important biochemical pathways involved in formation from planktonic state to the mature biofilm of *V. fischeri*.

Nominated altered chemicals (or biomolecules) were identified in both the planktonic and biofilm samples and are listed in Fig. 3 in a form of a heat map with a subset of color-coded metabolites (around 200) indicating critical fold changes between the two states. A concordance analysis of the two metabolic signatures (corresponding to the average of three analysis per condition, biofilm versus planktonic) indicates significant differences in metabolite profiles. Results for biofilm signatures indicate, up-regulated differences (2 fold) detected for multiple organic acids including carboxylic, phosphoric, aspartic, docosanoic, malonic, hydrobenzoic and keto-gluconic, as well as important sugars such as fructose, mannose, and maltose. Glycerol-derived components were also detected (hexadecanoglycerol, dodecanoglycerol, heptadecanoglycerol, and tetradecanoglycerol), and alcohols included mannitol and tetradecanol. Components that were observed to be significantly down-regulated (2–3 fold) in biofilms and

Table 3 Summary of *Vibrio fischeri* ETJB1H biofilm proteins identified by UPLC/MS

Reference[a]	Protein identified	Theoretical MW(KDa)/IP[b]	No. peptides matched	Coverage (%)
GI:59711008	UDP-N acetylglucosomine 1 carboxyvinyltransferase	44.72/5.28	20	60
GI:59479183	Outer membrane protein U porin OmpU	33.18/3.92	12	55
GI:59712357	Cob(I)yrinic acid a,c-diamide adenosyltransferase	28.31/4.08	10	53
GI:59710825	ABC transporter ATP-binding protein	72.70/4.50	22	48
GI:197317623	carbon starvation protein A	53.51/5.14	13	44
GI:59712372	ATP-dependent Clp protease ATP-binding subunit	82.58/4.8	31	40
GI:31414756	sigma 54 transcriptional activator	53.89/5.15	12	37
GI:59482580	multidrug efflux system protein	122.42/4.87	34	37
GI:59480318	phosphate-binding protein	29.45/5.00	8	36
GI:59710693	Oxidoreductase	43.69/5.25	10	36
GI:121308572	Bioluminescence regulatory protein	84.37/4.20	24	35
GI:172087731	Flagellin	48.09/4.05	9	35
GI:59711558	putative Holiday junction DNA helicase RuvA	29.78/4.71	9	35
GI:148536406	RecA protein	28.54/4.32	8	35
GI:59480175	chaperone, DnaK-like protein	69.88/4.52	10	35

[a]NCBI reference sequence
[b]Calculated using AnTheProt (http://antheprot-pbil.ibcp.fr/) and Scansite3 (http://scansite3.mit.edu/#home)

up-regulated in the planktonic state include threoic acid, hydroxypyrimidine, tyramine, and cellobiose (Fig. 3).

Discussion

In this study, we selected a meta-proteomics approach in order to resolve functional differences between the two life-history stages for *V. fischeri* (biofilms versus planktonic cells), and to describe factors that are important for successful colonization of the host, since the biofilm studied here resembles the community found in sepiolid squid light organs (for example, monospecificity, enriched environment, and availability of carbon sources), *Vibrio fischeri* biofilms and planktonic cells have been observed previously under Scanning Electron Microscopy [28–31]. There are distinct morphological differences; for example, planktonic cells are observed with flagellum and different shapes of fimbriae [28], whereas biofilms are observed as a flocculent bacterial mass encapsulated in a polysaccharide matrix [29, 31] structure that is similar to the one observed in biofilms formed in the squid's light organ [31].

Results indicate differences in protein expression levels, including the number of unique proteins detected, as well as those with enhanced levels of expression (Table 3). The two-dimensional approach used was limited to the inability to identify all the spots through mass spectrometry as previously reported [32]. Alternatively, identification of

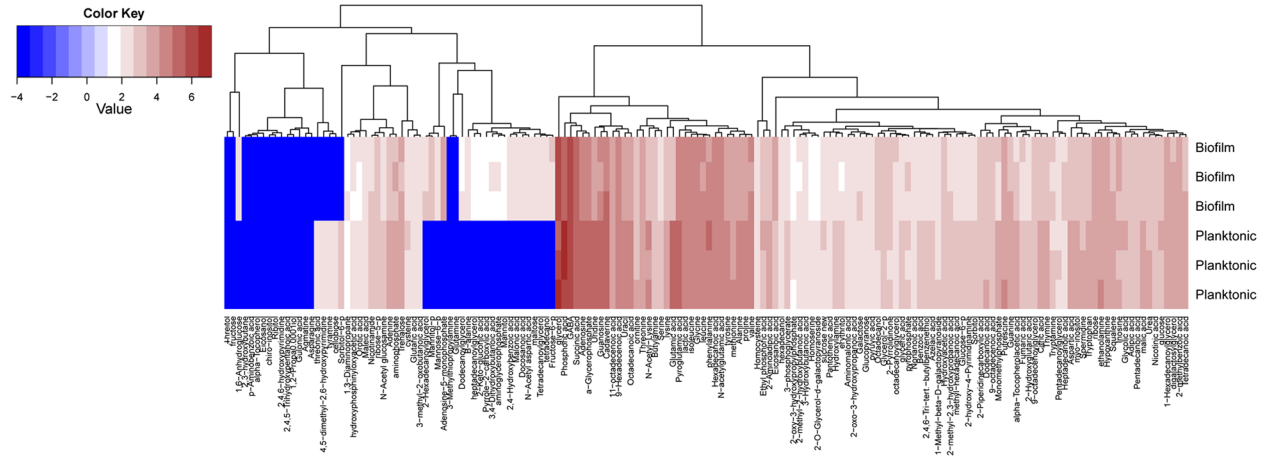


Fig. 3 Heat map indicating the metabolomic profile of biofilm and planktonic ETJB1H *Vibrio fischeri* strains. Data are shown in triplicate as a colored map reflecting the logarithms that relate to the metabolite changes (red areas indicate an increase in metabolite abundancy, blue areas depict a decrease). Log values are color coded as indicated in the scale

some proteins were achieved through shotgun proteomics (a combination of ultra-performance liquid chromatography and mass spectrometry or UPLC/MS), and allowed the comparison of both theoretical molecular weight and isoelectric points to experimental values from proteins identified in the two dimensional gel, which validated the combinational methodology. Proteins identified in this manner were then classified depending on their functions including: a) stress-response regulators, b) catalytic enzymes, c) transporters, d) metabolic enzymes, and e) structural proteins. Proteins that were previously reported overexpressed include those involved in energy generation (*e.g.*, succinyl-CoA synthase) and in biosynthesis (*e.g.*, ribosomal factors) [33–43]. Future studies will focus on more specific differences between these two community phenotypes (environmental versus host biofilms), since it has been observed that environmental versus symbiotic strains respond differently to stress, including fluctuations in temperature and salinity [25].

Upregulated proteins are important for maintenance and integrity of *V. fischeri* biofilms

In *Vibrio fischeri,* flagellin is expressed constitutively, and is esseantial for host colonization [39–41]. *V. fischeri* contains between one and five flagellar filaments that form a tuft of polar sheathed flagella [39]. *V. fischeri* flagellins are more similar to each other than to flagellins of other *Vibrio* species [38, 39], and therefore was not possible to differentiate the type of flagellin detected in this study. However, two proteins detected might be the closest match: (i) the flagellin FlgA that has been reported to be important for initial stages of host colonization [39] and (ii) FlgF, found to be important for host colonization and biofilm formation [39, 41].

Stress response proteins were also identified in this study. Elevated expression of the chaperone Clp was detected here. Clp proteins are known to regulate virulence in pathogenic bacteria such as *Porphyromonas gingivalis* [42] and *Vibrio cholerae* [43], and increased concentrations observed in *V. fischeri* biofilms might be related to increased success in host colonization, since it is believed that strong biofilm formers are also excellent host colonizers [1]. More interestingly, as reported for *V. cholerae*, Clp chaperone may be linked to the control of oxidative stress within the biofilm matrix (this would also include oxidoreductase, which was detected in this study). Oxidative stress is thought to be a result of a combination of slow growth in conjunction with a shift in oxygen at different depths of the biofilm [44]. Additional stress-related proteins were overexpressed in biofilms, including DnaK (molecular chaperone important for protein protection from denaturalization) [45] and the carbon starvation protein A (promotes peptide utilization during carbon starvation) [46]. It has been suggested that different micro-niches within the biofilm community are continuously exposed to various environmental stresses, inducing an increase of stress resistance mechanisms [47, 48].

Stress-inducible biofilm formation also produces DNA damage, which can trigger the bacterial SOS response initiated by the sensor protein RecA [49, 50], which was overexpressed in the biofilm samples. In addition, these methods detected the protein RuvA (responsible for Holiday junction formation as well as initiation of the SOS response). RuvA along with RuvB in the presence of ATP release the cruciform structure formed during strand exchange during homologous recombination [51], which might also occur in *Vibrio* biofilm communities. Since expression of proteins related to DNA repair can be synthesized up to 10 times more in biofilms [52, 53], this may result in undetectable traces in planktonic bacteria.

Another important component detected in *V. fischeri* biofilms is sigma factor 54 (σ^{54}) that has been reported to be an important regulator of a wide range of bacterial processes, including nitrogen metabolism in *Escherichia coli* [54], biogenesis of flagella in *Vibrio parahaemolyticus* and *Vibrio cholerae* [55, 56], and bioluminescence in *Vibrio harveyi* [57]. More interestingly, σ^{54} in *V. fischeri* is encoded by the *rpoN* gene [58] that is overexpressed during the biofilm state, controlling flagellar biosynthesis (motility), nitrogen assimilation, luminescence, and biofilm formation [58].

Biofilms cells expressed a presumptive ABC transporter, which corresponds to a major class of translocation machinery in multiple bacterial species [59, 60]. ABC transporters have been previously identified to be differentially expressed during biofilm formation in *Pseudomonas aeruginosa* and *E. coli* [61, 62] and they may be linked to the transport of small molecules and solutes during the formation and maintenance of the mature biofilm compared to the cells in their planktonic state. In addition, the detected ABC transporter might influence cytoplasmic pH homeostasis by increasing transmembrane fluctuation of ions (for example K+) to allow compensation after pH stress (or osmoprotection). It is known that the light organ of sepiolid squids undergoes anaerobic stress based on fermentation genes expressed solely in the light organ environment [16], and possible drops in pH may be due to the acid by-products accumulating during this time.

The outer-membrane protein detected in this study (OmpU) has been previously identified in symbiotic *V. fischeri*, and has an important role in the initiation of colonization of the squid light organ [37]. In addition, disruption of the *ompU* gene results in increased sensitivity to membrane-disrupting chemical agents such as chlorine and organic acids [37]. These observations indicate that OmpU might have an important role in maintaining membrane integrity during *V. fischeri* biofilm development by providing defense mechanisms that are essential for resistance to the acidic environment within the biofilm matrix.

Biofilm development is guided by several regulatory systems. One of the important processes is formation of the exopolyssacharide (EPS) matrix, a hallmark of bacterial biofilms. UDP-GlcNAc (UDP-N acetylglucosomine 1 carboxyvinyltransferase) was detected in this study, and has an important role in the synthesis of EPS by acting as a transcriptional regulator [63].

Phosphatase-binding proteins have been described to increase production of the second messenger cyclic diguanylic acid (c-di-GMP) in *Pseudomonas aeruginosa* [64] and this protein was prevalent in our study. C-di-GMP is a central regulator of the prokaryote biofilm lifestyle [65], including *V. fischeri* biofilms.

There are multiple proteins that regulate bioluminescence. In particular, biofilm formation and bioluminescence are linked through proteins that regulate bacterial communication or quorum sensing [66]. One protein detected in this study (bioluminescence regulatory protein) may up-regulate the quorum sensing cascade, which, among other functions, has been reported to increase production of EPS [66]. This finding is particularly important for host-related biofilms and host survival since bacterial bioluminescence (increased in high bacterial density, as in the case of biofilms and not planktonic cells) is the main process that sepiolid squids use for the counterillumination (silhouette reduction from the moonlight at night). Therefore, in *V. fischeri*, regulation of bioluminescence is activated when bacterial concentration significantly increases in number and proximity (in the case of biofilms) [66–68]. Provided that the bioluminescence protein was detected in a biofilm that was formed under laboratory conditions, a similar bacterial community (and protein expression) is present in the squid host.

Metabolomic profile revelas an increased number of components of the biofilm matrix

Most *Vibrio* biofilm matrices are composed of polysaccharides, such as the VPS (*Vibrio* polysaccharide), present in *V. cholerae* biofilms [1]. This metabolomic study detected carbohydrates that were present during the biofilm state and absent in planktonic cells. Proteins present consisted of mannose, maltose, fructose, and other monomeric sugars (galactose and glucose). In addition, smaller amounts of N-acetylglucosamine and N-acetyl glutamic acid were detected and have been described to be part of the VPS [69]. The presence of multiple glycerol-derived metabolites suggests that biofilm cells may use phospholipids released from neighboring cells, which possibly serve as a carbon source for amino acid biosynthesis. This metabolomic study revealed the presence of highly phosphorylated (and non-phosphorylated) glucans, which have been identified to be associated with the matrix of strains of *P. aeruginosa* [70]. Metabolites detected are important for synthesis of EPS components, or are related to regulatory processes involving second messengers (such as c-di-GMP). These components are strictly unique of community formation, but further research is required in order to determine if these metabolites are also important for mutualistic associations and what could be the metabolic differences between environmental and mutualistic biofilms. Future studies are needed in order to test whether glycerol and phosphate-derived components (detected in this study) are dominant in the squid's light organ and how these components may contribute to host specificity and maintenance of symbiosis integrity.

Conclusions

The objective of this study was to examine conserved proteomic and metabolomic signatures of both planktonic/free-living *V. fischeri* and their biofilm communities. Our results establish the methodology to utilize meta-proteomic analysis, enabling a more detailed perspective for understanding the biochemistry and metabolism of growth between the free-living/planktonic and community biofilm stages in a mutualistic bacterium. This meta-proteomic approach also improves the understanding of biofilms at a molecular level that is different from a transcriptomic or genomic comparisons (at the "functionality" level, which includes endpoint products, proteins, and metabolites). Results indicate a clear divergence associated with the restructuring of regulatory networks that allow community formation. Unique proteins and metabolites (mostly related to stress-responses, formation of the biofilm matrix and phosphorylated components) were significantly overexpressed in the biofilm state when compare to the free-living planktonic cells. Future work will entail combination of more differential studies (transcriptomics) to link the role of candidate genes to biochemical pathways and protein functionality.

Additional file

Additional file 1: Figure S1. 2D-PAGE gel of unique spots presents in protein exudates from A) *Vibrio fischeri* ETJB1H planktonic cells and B) *Vibrio fischeri* ETJB1H biofilm cells. Circles indicate the spots that are unique for each protein extraction. Spot detection revealed 271 spots for the planktonic cells and 199 spots for biofilm cells. Using planktonic cells as the reference profile, there were a total of 21 spots upregulated and 52 downregulated for the biofilm cells. (DOCX 656 kb)

Abbreviations

V. fischeri: *Vibrio fischeri*; ES114: *Euprymna scolopes* st. 114; ETJB1H: *Euprymna tasmanica* st. Jervis Bay 1H; 2D-PAGE: Two-dimensional PolyAcrylamide Gel Electrophoresis; EPS: Exopolysaccharide; MS: Mass Spectrometry; LC: Liquid Chromatography; LBS: Luria Broth high Salt; IPG: Immobilized pH Gradient; mA: Milliampere; DTT: Dithiothreitol; SDS: Sodium Dodecyl Sulfate; FT-ICR: Fourier Transform-Ion Cyclotron Resonance; Da: Dalton; KDa: Kilodalton; FDR: False Discovery Rate; Q-TOF: Quadruple-Time of Flight; UPLC: UltraPerformance Liquid Chromatography; MW: Molecular Weight; IP: Isoelectric Point; c-di-GMP: Cyclic diguanylate; VPS: *Vibrio* Polysaccharide.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Conceived and designed the experiments: M.K.N., A.C.-D. Performed the experiments: A.C.-D., C.G. Analyzed the data: A.C.-D. Contributed materials/analysis tools: M.K.N. Wrote the paper: M.K.N., A.C.-D. Final approval of manuscript: M.K.N., A.C.-D., C.G. All authors read and approved the final manuscript.

Authors' information
Not applicable.

Acknowledgements
We thank Dr. Augustine A. DiNovo from Guil Biosciences Company for his assistance with the 2D Page electrophoresis proteomic analysis, and Dr. Lucas Li from the Roy J. Carver Biotechnology Center (CBC) at the University of Illinois for the Metabolomic analysis.
A.C.-D. was supported by RISE (NIH NIGMS R25GM061222), NASA (NMSGC) and the IDIP T32 fellowship AI007538 at the University of New Mexico. C.G. was supported by the NMSU NIH MARC program (NIH-GM0007667-34). This project was partially supported by NSF IOS-0744498, NIH NIAID 1SC1AI081659-01, and NIH ARRA-3SC1AI081659-02S1 to M.K.N.

Received: 9 May 2015 Accepted: 8 October 2015
Published online: 23 October 2015

References

1. Yildiz F, Visick K. *Vibrio* biofilms: so much the same yet so different. Trends in Microbiol. 2008;17(3):109–18.
2. Dopheide A, Lear G, Stott R, Lewis G. Relative diversity and community structure of ciliates in stream biofilms according to molecular and microscopy methods. Appl Environ Microb. 2009;75:5261–72.
3. Kim T, Sohn S, Kim H, Lee S. Strategies for systems-level metabolic engineering. J Biotechnol. 2008;3:612–23.
4. Kim S, Wei C. Molecular characterization of biofilm formation and attachment of *Salmonella enterica* serovar *typhimurium* DT104 on food contact surfaces. J Food Prot. 2009;72(9):1841–7.
5. Price L, Liu C, Melendez J, Frankel Y, Engelthaler D, Aziz M, et al. Community Analysis of Chronic Wound Bacteria Using 16S rRNA Gene-Based Pyrosequencing: Impact of Diabetes and Antibiotics on Chronic Wound Microbiota. PLoS ONE. 2009;4(7):1.
6. Resch A, Leicht S, Saric M, Pasztor L, Jakob A, Gotz F, et al. Comparative proteome analysis of *Staphylococcus aureus* biofilm and planktonic cells and correlation with transcriptome profiling. Proteomics. 2006;6(6):1867–77.
7. Jones D, Albrecht D, Dawson K, Schaperdoth I, Freeman K, Pi Y, et al. Community genomic analysis of an extremely acidophilic sulfur-oxidizing biofilm. ISME. 2012;6(1):158–70.
8. Neufeld JD, Wagner M, Murrel JC. Who eats what, where and when? Isotope-labeling experiments are coming of age. ISME. 2007;1(2):103–10.
9. Steinberg C. The actual response: ecological proteomics and metabolomics. Stress Ecology pp. 2012;241–278.
10. Patrauchan M, Sarkisova S, Franklin M. Strain-specific proteome responses of *Pseudomonas aeruginosa* to biofilm-associated growth and to calcium. Microbiology. 2007;153(11):3838–51.
11. Booth SC, Workentine ML, Wen J, Shaykhutdinov R, Vogel HJ, Ceri H, et al. Differences in metabolism between the biofilm and planktonic response to metal stress. J Proteome Res. 2011;10(7):3190–9.
12. Kleiner M, Wentrup C, Lott C, Teeling H, Wetzel S, Young J, et al. Metaproteomics of a gutless marine worm and its symbiotic microbial community reveal unusual pathways for carbon and energy source. Proc Natl Acad Sci USA. 2012;109(19):E1173–82.
13. Serra DO, Lucking G, Weiland F, Schulz S, Gorg A, Yantorno OM, et al. Proteome approaches combined with Fourier transform infrared spectroscopy revealed a distinctive biofilm physiology in *Bordetella pertussis*. Proteomics. 2008;8(23–24):4995–5010.
14. Wilmes P, Remis JP, Hwang M, Auer M, Thelen MP, Banfield JF. Natural acidophilic communities reflect distinct organismal and functional organization. The ISME J. 2009;3:266–70.
15. Booth S, George IAS, Zannoni D, Cappelletti M, Duggan GE, Ceri H, Turner RJ (2013) Effect of aluminum and copper on biofilm development of *Pseudomonas pseudoalcaligenes* KF707 and *P. fluorescens* as a function of different media compositions. Metallomics. doi:10.1039/C3MT20240B
16. Jones BW, Nishiguchi MK. Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). Marine Biol. 2004;144:1151–5.
17. Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK. Spatial and temporal distribution on the Vibrionaceae in costal waters of Hawaii, Australia and France. Microbial Ecol. 2007;54:314–23.
18. Nyholm SV, McFall-Ngai MJ. The winnowing: establishing the squid-*Vibrio* symbiosis. Nature. 2004;2:632–42.
19. Fidopiastis P, Miyamato C, Jobling M, Meighen E, Ruby E. LitR, a new transcriptional activator in *Vibrio fischeri*, regulates luminescence and symbiotic light organ colonization. Mol Microbiol. 2002;45:131–43.
20. Fidopiastis P, Von B, Ruby E. A new niche for *Vibrio logei*, the predominant light organ symbiont of the Mediterranean sepiolid squids *Sepiola affinis* and *S. robusta*. J Bacteriol. 1998;180:59–64.
21. Nishiguchi MK. Temperature affects species distribution in symbiotic populations of *Vibrio*. Appl Environ Microbiol. 2000;66:3550–5.
22. Guerrero-Ferreira RC, Gorman C, Chavez-Dozal A, Wille S, Nishiguchi MK. Characterization of the bacterial diversity of Indo-West Pacific loliginid and sepiolid squid light organs. Microb Ecol. 2012;65:214–22.
23. Jones BW, Lopez JE, Huttenberg J, Nishiguchi MK. Population structure between environmentally transmitted *Vibrios* and bobtail squids using nested clade analysis. Mol Ecol. 2006;15:4317–29.
24. Merritt JH, Kadouri DE, O'Toole GA (2005) Growing and analyzing static biofilms. Curr Protocols Biol. 1B.1.1-1.1.17. doi:10.1002/9780471729259.mc01b01s00.
25. Chavez-Dozal A, Nishiguchi MK. Effect of environmental factors on biofilm formation by *Vibrio fischeri* isolates. J Basic Microbiol. 2011;5:452–8.
26. Doino Lemus J, McFall-Ngai M. Alterations in the proteome of the *Euprymna scolopes* light organ in response to symbiotic *Vibrio fischeri*. Appl Environ Microbiol. 2000;66(9):4091–7.
27. Kayser JP, Vallet JL, Cerny RL. Defining parameters for homology-tolerant database searching. J Biomol Tech. 2004;15(4):285–95.
28. Nair VN, Nishiguchi MK. Biological properties (in vitro) exhibited by free-living and symbiotic *Vibrio* isolates. Vie et Milieu. 2009;59:277–85.
29. Ariyakumar DS, Nishiguchi MK. Characterization of two host-specific genes, mannose sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. FEMS Microbiol Lett. 2009;299:65–73.
30. Chavez-Dozal A, Hogan D, Gorman C, Quintanal-Villalonga A, Nishiguchi MK. Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. FEMS Microbial Ecol. 2012;81(3):562–73.
31. Nyholm SV, Nishiguchi MK. The evolutionary ecology of a sepiolid squid-*Vibrio* association: From cell to environment. Vie et Milieu. 2008;58:175–84.
32. Gauci VJ, Wright EP, Coorssen JR. Quantitative proteomics: assessing the spectrum of in-gel protein detection methods. J Chem Biol. 2011;4(1):4–29.
33. Kalmokoff M, Lanthier P, Tremblay TL, Foss M, Lau PC, Sanders G, et al. Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. J Bacteriol. 2006;188(12):4312–20.
34. Sanchez CJ, Kumar N, Lizcano A, Shivshankar P, Hotopp JCD, Jorgensen JH, et al. *Streptococcus pneumoniae* in biofilms are unable to cause invasive disease due to altered virulence determinant production. PLoS ONE. 2011;6(12):e28738.
35. Williamson KS, Richards LA, Perez-Osorio AC, Pitts B, McInnerney K, Stewart PS, Franklin MJ (2012) Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. J Bacteriol 194(9): doi:10.1128/JB.00022-12
36. Guerrero-Ferreira R, Nishiguchi MK. Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches. Environ Microbiol Rep. 2010;2(4):514–23.
37. Aeckersberg F, Lupp C, Feliciano B, Ruby EG. *Vibrio fischeri* outer membrane protein OmpU plays a role in normal symbiotic colonization. J Bacteriol. 2001;183(22):6590–7.
38. Klose KE, Mekalanos JJ. Differential regulation of multiple flagellins in *Vibrio cholerae*. J Bacteriol. 1998;180(2):303–16.
39. Millikan DS, Ruby EG. Flagellin A is essential for normal motility and for symbiotic competence during initial squid light organ colonization. J Microbiol. 2004;186:4315–25.

40. Millikan DS, Ruby EG. Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. Appl Environ Microbiol. 2002;68:2519–28.
41. Chavez-Dozal A, Hogan D, Gorman C, Quintanal-Villalonga A, Nishiguchi MK. Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. FEMS Microbial Ecol. 2012;81(3):562–73.
42. Capestany CA, Tribble GD, Maeda K, Demuth DR, Lamont RJ. Role of the Clp system in stress tolerance, biofilm formation, and intracellular invasion in *Porphyromonas gingivalis*. J Bacteriol. 2008;190(4):1436–46.
43. Nag S, Das S, Chaudhury K. *In vivo* induced clpB1 gene of *Vibrio cholerae* is involved in different stress responses and affects *in vivo* cholera toxin production. Biochem Biophys Res Commun. 2005;331(4):1365–73.
44. Boles BR, Singh PK. Endogenous oxidatives stress produces diversity and adaptability in biofilm communities. Proc Natl Acad Sci USA. 2008;105(34):12503–8.
45. Gotoh H, Zhang Y, Dallo SF, Hong S, Kasaraneni N, Weitao T. *Pseudomonas aeruginosa*, under DNA replication inhibition, tends to form biofilms via Arr. Res Microbiol. 2008;159(4):294–302.
46. Schultz JE, Matin A. Molecular and functional characterization of a carbon starvation gene of *Escherichia coli*. J Mol Biol. 1991;218(1):129–40.
47. Bernier SP, Lebeaux D, DeFrancesco AS, Valomon A, Soubigou G, Coppee JV, et al. Starvation, togheter with the SOS response, mediates high biofilm specific tolerance to the fluoroquinolone ofloxacin. PLoS Genet. 2013;9(1):e10033144.
48. Costerton JW, Lewadowski DE, Caldwell DR, Korber DR, Lappin-Scott HM. Microbial biofilms. Annual Rev Micro boil. 1995;49:711–45. ISSN 0066–4227.
49. Gotoh H, Zhang Y, Dallo SF, Hong S, Kasaraneni N, Weitao T. *Pseudomonas aeruginosa*, under DNA replication inhibition, tends to form biofilms via Arr. Res Microbiol. 2008;159(4):294–302.
50. Uranga LA, Balise VD, Benally CV, Grey A, Lusetti SL. The *Escherichia coli* DinD protein modulates RecA activity by inhibiting postsynaptic RecA filaments. J Biol Chem. 2011;286(34):29480–91.
51. Van Gool AJ, Hajibagheri NMA, Stasiak A, West SC. Assembly of the *Escherichia coli* RuvABC resolvasome directs the orientation of holliday junction resolution. Gen Develop. 1999;13:1861–70.
52. Marks LR, Reddinger RM, Hakansson AP. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in *Streptococcus pneumoniae*. mBio. 2012;3(5):e00200–12.
53. Pysz MA, Conners SB, Montero CI, Shocley KR, Johnson MR, Ward DE, et al. Transcriptional analysis of biofilm formation processes in the anaerobic hyperthermophilic bacterium *Thermotoga maritima*. Appl Environ Microbiol. 2004;70(10):6098–112.
54. Reitzer L, Schneider BL. metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. Microbiol Mol Biol Rev. 2001;65(3):422–44.
55. Stewart BJ, McCarter LL. *Vibrio parahaemolyticus* FlaJ, a homologue of FliS, is required for production of a flagellin. Mol Microbiol. 1996;20:137–49.
56. Prouty MG, Correa NE, Klose KE. The novel sigma54 and sigma 28-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. Mol Microbiol. 2001;39(6):1595–609.
57. Lilley BN, Bassler BL. Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. Mol Microbiol. 2000;36(4):940–54.
58. Wolfe AJ, Millikan DS, Campbell JM, Visick KL. *Vibrio fischeri* sigma-54 controls motility, biofilm formation, luminescence, and colonization. Appl Environ Microbiol. 2004;70(4):2520–4.
59. Tomii K, Kanehisa K. A comparative analysis of ABC transporters in complete microbial genomes. Genome Res. 1998;8:1048–59.
60. Cuthbertson L, Kos V, Whitfield C. ABC transporters involved in export of cell surface glycoconjugates. Micorbiol Mol Biol Rev. 2010;74(3):341.
61. Hentzer M, Eberl L, Givskov M. Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation. Biofilms. 2005;2:37–61.
62. Whitfield C. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu Rev Biochem. 2006;75:39–68.
63. Steiner S, Lori C, Boehm A, Jenal U. Allosteric activation of exopolysaccharide synthesis through cyclic di-GMP-stimulated protein-protein interaction. The EMBO J. 2013;32:354–68.
64. Ueda A, Wood TK. Connecting quorum sensing, ci-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TbpA (PA3885). PLoS Pathog. 2009;5, e1000483.
65. Cotter PA, Stibitz S. c-di-GMP mediated regulation of virulence and biofilm formation. Curr Opin Microbiol. 2007;10(1):17–23.
66. Hammer BK, Bassler BL. Quorum sensing controls biofilm formation in *Vibrio cholerae*. Mol Microbiol. 2003;50(1):101–4.
67. Peat SM, Adams BJ. Natural selection on the *luxA* gene of bioluminescent bacteria. Symbiosis. 2008;46:101–8.
68. Kierek K, Watnick PI. Environmental determinants of *Vibrio cholerae* biofilm development. Appl Environ Microbiol. 2003;69(9):5079–88.
69. Fong JCN, Syed KA, Klose KE, Yildiz FH. Role of *Vibrio* polysaccharide (*vps*) genes in VPS production, biofilm formation and *Vibrio cholerae* pathogenesis. Microbiol. 2010;156(9):2757–69.
70. Coulon C, Vinogradov E, Filloux A, Sodovskaya I. Chemical analysis of cellular and extracellular carbohydrates of a biofilm-forming strain *Pseudomonas aeruginosa* PA14. PLoS ONE. 2010;5(12), e14220.

VIE ET MILIEU - LIFE AND ENVIRONMENT, 2015, 65 (4): 187-200

GENOMICS OF AN ENVIRONMENTALLY-TRANSMITTED SYMBIOSIS: NEWLY SEQUENCED *VIBRIO FISCHERI* GENOMES FROM DIFFERENT HOST SQUIDS AND GEOGRAPHIC LOCATIONS

J. H. HOWARD[1], B. L. WILLIAMS[2], M. BREEN[3], M. K. NISHIGUCHI[*4]*

[1] Pennsylvania State University-Abington, Department of Biology, 1600 Woodland Road, Abington, Pennsylvania 19001-3990, USA
[2] Utah State University Uintah Basin, Department of Biology, 320 N 2000 W (Aggie Blvd), Vernal, Utah 84078-4228, USA
[3] Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, University Rd, Southampton SO17 1BJ, United Kingdom
[4] New Mexico State University, Department of Biology, BOX 30001, MSC 3AF, Las Cruces, New Mexico 88003-8001, USA
** Corresponding author: nish@nmsu.edu*

VIBRIO FISCHERI
GENOME
SYMBIOSIS
BOBTAIL SQUID
EUPRYMNA
SEPIOLA

ABSTRACT. – Environmentally-transmitted symbiotic bacteria must balance selective pressures of host specificity and the abiotic environment. Here, we investigate genomes of three strains of *Vibrio fischeri* isolated from three different squid species living in various temperature and salinity environments. These sequences were compared to others from symbiotic and free-living vibrios for gene orthology, functional subsystem, selection, and phylogenetic analyses. A *V. cf. campbellii* bacterium isolated from seawater also was sequenced and provided an outgroup for the selection and phylogenetic analyses. This investigatory study provides the basis for further directed studies that may elucidate the genetic architecture underlying adaptation to both the free-living environment and host squids in *V. fischeri*. Symbiotic vibrios in this study had relatively conserved genomes with evidence of strong purifying selection and few duplication events. Horizontal gene transfer may provide a mechanism for the acquisition of host and environment-specific genes. Despite spending a majority of time in the free-living stage and a low probability of host-colonization for any one bacterium, symbiotic *V. fischeri* appear to have evolutionary histories molded by their squid hosts.

INTRODUCTION

Environmentally-transmitted symbiotic bacteria must be able to survive both within and external to their specific host. In opposition to vertically transmitted symbioses, hosts are axenic at birth and acquire symbionts from the environment. How symbionts actually balance these two environments has broad implications for pathogen transmission, coevolutionary theory, cooperation, and stability of symbiosis dynamics (Nishiguchi *et al*. 2008, Nyholm & Nishiguchi 2008, Sachs & Simms 2006, Sachs *et al*. 2011). Attempts to map the genetic basis of adaptation to either free-living or host environment have greatly increased with the ability to rapidly sequence whole genomes (Hudson 2008, Nadeau & Jiggins 2010, Stapley *et al*. 2010). For example, genetic signatures for pathogenicity in a *Streptococcus* strain have been identified (Suzuki *et al*. 2011), as have differences in metabolic capabilities in rumen symbionts *Prevotella* (Purushe *et al*. 2010), differences in cold tolerance in *Neurospora* (Ellison *et al*. 2011), and phosphorus acquisition in phosphorus-limited environments in *Pelagibacter* and *Prochlorococcus* (Coleman & Chisholm 2010). Not only can influential point mutations be identified, but whole genome patterns, such as duplication events, expansion or reduction of the core genome, composition of the accessory genome, and rearrangement of regulatory elements can be identified and investigated for their role in adaptation (Gilad *et al*. 2009, Levasseur & Pontarotti 2011, Medina & Sachs 2010).

We investigated an environmentally-transmitted symbiosis between bobtail squids and their *Vibrio* symbionts because of the breadth of host species and environmental conditions, as well as the wealth of prior knowledge available about this symbiotic complex. We examined three strains of *V. fischeri* isolated from different host species of squid from different geographic locations to catalog the genetic diversity of this complex system as a launching point for more in-depth comparative studies that might pinpoint the functional significance of genetic elements involved in adaptation to either host or free-living environment.

*The sepiolid squid-*Vibrio *model system*

Bobtail squids (Cephalopoda: Sepiolidae) harbor bioluminescent bacterial symbionts (γ-Proteobacteria: Vibrionaceae) in a specialized organ that directs light downwards to mask the squids' silhouette from benthic predators (Jones & Nishiguchi 2004). Axenic squid hatchlings are colonized through a "sieve" of host defenses that selects for only coevolved mutualism-competent strains (Nyholm & McFall-Ngai 2004). The squid and their colonists then enter a diel cycle (Boettcher *et al*. 1996), whereby the vibrios first grow and populate the light organ during the day while the squid is quiescent and hidden in the

sand. During the evening the squid emerges and bacterial bioluminescence, which is triggered by quorum sensing that occurs at high bacterial densities (Eberhard 1972, Fuqua *et al*. 1994, Nealson 1977), is directed ventrally by the squid to match downwelling moonlight (Jones & Nishiguchi 2004). Upon sunrise, the squid vents 90 % of the bacteria (Graf & Ruby 1998). The venting process seeds the surrounding seawater with a high number of potential vibrio symbionts capable of colonizing newly-hatched squids (Lee & Ruby 1994, Ruby & Lee 1998). Thus, symbiotic *Vibrio* must navigate both the host and free-living environments.

Coevolution of the symbionts and hosts has influenced colonization success. Some bacterial strains can efficiently colonize multiple squid species, as observed in the Mediterranean Sea where sympatric squid species live (Nishiguchi 2000, Nishiguchi *et al*. 2008, Nyholm & McFall-Ngai 2004, Nyholm & Nishiguchi 2008, Ruby 2008), while others are more host-specific, as demonstrated in the Indo-West Pacific where there is no geographical overlap among squid species (Nishiguchi 2000, Jones *et al*. 2006, Jones *et al*. 2007, Soto *et al*. 2009a, Zamborsky & Nishiguchi 2011). Fine sequence variation in genes required for the mutualism affect host specificity (Chavez-Dozal *et al*. 2014). In competition experiments using *Vibrio* isolates from different squid species, squids show preference for their native bacterial strains, and a hierarchy of competitive dominance exists (Chavez-Dozal *et al*. 2014, Jones *et al*. 2006, Nishiguchi *et al*. 1998, Nishiguchi 2002, Soto *et al*. 2014). Such dominance can be conferred by genes of major effect, which can result in rapid fixation or alter the evolutionary trajectory of a lineage (Nadeau & Jiggins 2010, Orr 2005). For example, introduction of a single regulatory gene (*rscS*) to a strain of *V. fischeri* symbiotic with a fish conferred the ability to colonize a novel host species (bobtail squid); this gene may have played a role in host-switching in this mutualism complex (Mandel *et al*. 2009). Experimentally evolving a strain native to *E. scolopes* in another squid species (*E. tasmanica*) resulted in an increase in colonization efficiency and competitive ability against native *E. tasmanica* strains in under 500 generations (Soto *et al*. 2012). Additionally, experimentally evolving both a free-living strain and a fish symbiotic strain in a squid species leads to a decrease in bioluminescense similar to what native strains exhibit (Schuster *et al*. 2010). In summary, strains appear to be uniquely adapted to their native squid hosts.

Mutualistic strains also illustrate adaptation to their free-living environment. Temperature and salinity tolerance varies among symbiotic strains of vibrios and matches native environmental conditions, which differ geographically (Soto *et al*. 2009a, 2009b). Adaptation to environmental variables can upset host-symbiont dynamics, e.g., native symbionts that are better adapted to colonize their host squid in laboratory settings may not be the most likely to colonize squids in nature, depending on the environmental conditions (Soto *et al*. 2012). In other words, strains that persist in high abundance because of adaptation to the free-living environment can colonize host squids even if other strains illustrated competitive dominance in a laboratory setting (Nishiguchi *et al*. 1998, Nishiguchi 2002, Jones *et al*. 2006, Nyholm & Nishiguchi 2008, Wollenberg & Ruby 2009, Soto *et al*. 2012). Similarly, abiotic factors and geography affect patterns of genetic diversity and colonization competency of *V. cholerae* in human hosts (Keymer *et al*. 2007). For symbionts, arriving at the host early can outweigh weaker colonization ability. In contrast, pleiotropic or epistatic responses in salinity tolerance occurred during experimental evolution in a novel host squid that may or may not be adaptive depending on the environment (Soto *et al*. 2012). Thus, the selective regime of free-living environment clearly produces adaptive responses in symbionts, but confounding variables may maintain the diversity of symbiotic strains that colonize any given host squid and thus also maintain diversity within the population of symbionts.

Study aims

The sepiolid squid-*Vibrio* symbiosis is well suited to explore the genetic scaffolding that enables adaptation to either the host or free-living environment. This well-studied model system has already revealed genetic mechanisms underlying adaptive phenotypes, such as several genes and regulatory elements involved in biofilm formation, which is necessary for host colonization (Yildiz & Visick 2009, Morris & Visick 2010, Chavez-Dozal & Nishiguchi 2011, Chavez-Dozal *et al*. 2012, Chavez-Dozal *et al*. 2014). Some genes and promoters involved in response to nitric oxide (NO) have been found using this model system. NO is a host-derived signal that may function as a population control strategy against the bacterial symbionts (Davidson *et al*. 2004, Wang *et al*. 2010). Other genes involved in circumventing host defenses or facilitating persistence in the free-living environment have been revealed by several transcriptional studies (Chun *et al*. 2008, Guerrero-Ferreira & Nishiguchi 2010, Jones & Nishiguchi 2006, Wier *et al*. 2010). These genet-

Table I. – Strain designations and ecological data for *Vibrio* species whose genomes were sequenced in this study.

Strain	Host	Locality	Temp	Temp °C	Salinity	Salinity ppt
CB37	None	SE Australia	Med	12–25	Med	20.0-35.5
ETJB5C	*Euprymna tasmanica*	SE Australia	Med	12–25	Med	20.0-35.5
EM17	*Euprymna morsei*	Japan	Low	2–17	Low	32.2-34.0
SA1G	*Sepiola affinis*	Mediterranean	Med	12–24	High	37.0-38.0

ic mechanisms appear to play parallel roles in other symbioses as well. Therefore, we used the natural diversity in squid hosts and environments to compare three mutualistic strains from three host squid species, from two temperature ranges, and three salinity levels (Table I). This allowed us to identify unique genes from each genome, differences in functional subsystems, genes currently under selection, point mutations in homologs, and investigate phylogenetic relationships among symbionts. Our findings will enable further studies targeting these points of interest with increased sample sizes and comparative methods focused on one variable (e.g., host, temperature, or salinity).

MATERIALS AND METHODS

Four bacterial genomes were sequenced via 454-pyrosequencing (Supplementary Table I). We chose this platform because longer reads and coverage depth are advantageous for assembly of small genomes like those of bacteria (Hudson 2008, Mardis 2008, Metzker 2010). Three strains of *V. fischeri* were isolated from different geographically located squid hosts (EM17 from *Euprymna morsei* in Tokyo Bay, Japan; ETJB5C from *E. tasmanica* in Jervis Bay, Australia; and SA1G from *Sepiola affinis* in Banyuls-sur-Mer, France; Table I). For comparison, we also sequenced a free-living, non-symbiotically competent *Vibrio* strain, *V. cf. campbellii* (CB37 isolated from Coogee Bay, Australia; Table I) and used it as our outgroup for phylogenetic and selection analyses. High-quality reads were obtained; sequence reads smaller than 40 nucleotides were discarded from analyses (Supplementary Tables I, II).

Pairwise and site-wise comparisons of homologs were completed and core and accessory genomes were identified (orthology analysis; Supplementary Table III). Analysis of these data enabled us to generate a list of genes potentially involved in adaptation to either the symbiotic or free-living state. Comparisons were made with other published genomes for orthology (symbiotic *V. fischeri* strains only) and selection analyses: ES114, a symbiont of *E. scolopes* (Ruby *et al*. 2005); MJ11, a symbiont of the fish *Monocentris japonica* (Mandel *et al*. 2009); SR5, a symbiont of a Mediterranean squid *Sepiola robusta* (Gyllborg *et al*. 2012); *V. campbellii* ATCC BAA-1116, a *Vibrio* strain isolated from a green barrel tunicate (Bassler *et al*. 2007, Lin *et al*. 2010); and two other vibrios, *Photobacterium profundum* 3TCK (Bartlett *et al*. 2006) and *Vibrio* sp. EJY3 (Roh *et al*. 2012). For phylogenetic analyses, we included our four sequenced genomes, and *V. fischeri* SR5 and MJ11.

Orthology analysis of the amino acid sequences from each *Vibrio* strain was performed using OrthoMCL database (Chen *et al*. 2006). Putative proteins encoded by the sequences were categorized as orthologs, paralogs, or orphans. Proteins failing to fall into clusters were analyzed with less stringent parameters (e-value of $1e^{-5}$ and minimum coverage of 50 %) using NCBI's BlastClust package and applying a reciprocal pBlast approach. Clusters were aligned with MUSCLE (Edgar 2004) using default parameters. We identified core clusters, defined as one orthologous sequence per strain, to calculate nucleotide diversity (π) and construct a phylogeny. The parameter Π, measured as the number of nucleotide differences between strains divided by the total length of a nucleotide sequence, was calculated for each set of orthologs in pairwise comparisons. We then averaged the values for all sets of pairwise comparisons to calculate the degree of polymorphism between strains. To visualize results identifying core and accessory genomes, we implemented PERL scripts within the publicly available Venn Diagram tool from VIB / UGent (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Annotated genetic elements were assigned to functional groups such as "Virulence, Disease, and Defense," "Cell Wall and Capsule," and "Stress Response" using Rapid Annotation Using Subsystem Technology (RAST) (Overbeek *et al*. 2014). The number of genes in each subsystem were summarized to highlight differences in allocation to subsystems among our symbionts, a model organism (ES114; (Ruby *et al*. 2005)), and the free-living *Vibrio* strain we sequenced. Annotated genes are provided in Supplementary Table IV.

The proportions of GC content in each open reading frame (ORF) were calculated, including the minimum, maximum, and SD of GC content per genome. Any ORF with GC proportions outside of ± 2 SDs of the mean were noted (Supplementary Table V). Normality of the distribution of GC proportions in the ORFs per genome was examined with Shapiro-Wilk tests.

The Codeml program was implemented from the PAML 4 package (Yang 2007) in a batch mode process on all clusters containing two or more strains to calculate selection ratios, both pairwise and sitewise, on all orthologs. Proteins and sites with Ka/Ks ratios exceeding 1.0 were classified as being under positive selection.

Maximum likelihood phylogenetic analysis was performed on multiple sequence alignments constructed at the protein level then concatenated to form orthologous alignments for the maximum likelihood program PhyML 3.0 (Guindon *et al*. 2010). We adopted the LG amino acid replacement matrix model (Le & Gascuel 2008) and utilized the SPR option, which provides the slowest but most accurate tree-topology search. A bootstrap analysis using 100 replicates also was performed.

RESULTS

Quality of sequence reads

We performed Roche 454 sequencing on the genomes of three *Vibrio fischeri* squid light organ isolates and on a free-living *Vibrio cf. campbellii* strain to increase our understanding of genetic differences that underlie symbionts of different squid species and different marine environments. Genomic DNA was sequenced in NMSU's sequencing facility as described in the Methods. Sequence read lengths of less than 40 nt were discarded. After standard filtering programs were implemented, we obtained good quality reads ranging from 47.9 % (EM17)

Table II. – Strain designations and information for *Vibrio* spp. and a *Photobacterium* sp. whose genomes were obtained from GenBank and used in orthology, positive selection, and sequence comparison analyses.

Species	Strain	Host	Geography	Accession No(s).	Reference
V. fischeri	ES114	*Euprymna scolopes*	Hawaii	NC_006840.2 NC_006841.2 NC_006842.1	(Ruby *et al.* 2005)
V. fischeri	MJ11	*Monocentris japonica*	Sea of Japan	NC_011184.1 NC_011185.1 NC_011186.1	(Mandel *et al.* 2009)
V. fischeri	SR5	*Sepiola robusta*	Mediterranean	CM001400.1 CM001401.1	(Gyllborg *et al.* 2012)
V. harveyi	ATCC BAA-1116	Free-living	Unknown	NC_009783.1 NC_009784.1	(Bassler *et al.* 2007)
Vibrio sp.	EJY3	*Grapsidae* (crab)	South Korea	NC_016613.1 NC_016614.1	(Roh *et al.* 2012)
P. profundum	3TCK	Free-living	San Diego Bay	NZ_AAPH00000000.1	(Bartlett *et al.* 2006)

to 78.4 % (ETJB5C), and averaging 59.0 %, of raw data, with an average read length of 480 nt (Supplementary Table II).

Comparison of Roche 454 sequencing reads to reference genomes

Reference genomes are provided in Table II. The reference genomes used to map sequences from the three *Vibrio fischeri* strains included in this genome-sequencing project were those of *V. fischeri* ES114 (Ruby *et al*. 2005) and *V. fischeri* MJ11 (Mandel *et al*. 2009). Genomic data from *V. campbellii* ATCC BAA-1116 (Lin *et al*. 2010), which originally was identified in GenBank data as a *V. harveyi,* was used as a reference for genome sequencing of the out-group used in analyses, *V. cf. cambellii* CB37. Our three symbiotic strains of *V. fischeri* have an average of 3898 genes, demonstrating slightly reduced genomes compared to an average genome size of 5173 genes found in the other non-*V. fischeri* vibrios used in our analysis (Table II). Overall, genes located on Chromosome I of the symbiotic strains were more conserved than genes located on Chromosome II. On average, the symbiotic strains shared 94.55 % sequence identity with *V. fischeri*'s Chromosome I, while Chromosome II averaged 89.89 %. The plasmid content carried in the symbiotic genomes is extremely variable. Only 20.06 % of the genes in ES114's plasmid are found in the plasmid of the Australian isolate, ETJB5C; roughly half as many are found in the Mediterranean isolate SA16, and none are present in the plasmid of the Japanese Sea isolate, EM17. Instead, the latter two strains share approximately 90 % of their plasmid content with the fish symbiont (MJ11) plasmid, while a very small percentage (less than 2 %) of ETJB5C's plasmid is the same as in MJ11 (Supplementary Table I).

Table III. – Nucleotide diversity (π) of our three symbiotic *V. fischeri* strains and free-living strain CB37, including comparisons to previously-sequenced *V. fischeri* strains ES114, MJ11, and SR5.

	SR5	ETJB5C	MJ11	SA1G	EM17	ES114	CB37
SR5	–	0.0296	0.0286	0.0291	0.0277	0.0301	0.2882
ETJB5C		–	0.0344	0.0364	0.0191	0.0185	0.2883
MJ11			–	0.0176	0.0332	0.0354	0.2883
SA1G				–	0.0359	0.0382	0.2883
EM17					–	0.0195	0.2884
ES114						–	0.2885
CB37							–

Orthology analysis

We identified the genes shared among all of the six genomes, thereby identifying the core *V. fischeri* genome. Including our three newly sequenced genomes, as well as the three reference genomes (ES114 (Ruby *et al*. 2005), SR5 (Gyllborg *et al*. 2012), and MJ11 (Mandel *et al*. 2009)), there are 3018 genes common, listed in Supplemental Table III. This represents 78.4 % of the average genome size. Eliminating the MJ11 genome from analysis increases the core genome for squid symbionts to 3091, whereas eliminating SR5 but leaving MJ11 in increases the core genome size to 3138. Our three newly sequenced genomes alone share 3186 orthologous genes. The overlap in genomic content among different groups of *V. fischeri* strains, along with *V. cf. campbellii* CB37, is depicted in Venn diagrams (Fig. 1). Accessory genomes of all six symbiotic strains, which include strain-specific genes as well as orthologs shared by some but not all of the symbiotic strains, contain on average 833 genes, with each strain possessing an average of 226 unique genes. Orthologs found in more than two but less than six genomes also include varying numbers of paralogous genes, ranging from 20 genes that have undergone at least one duplication

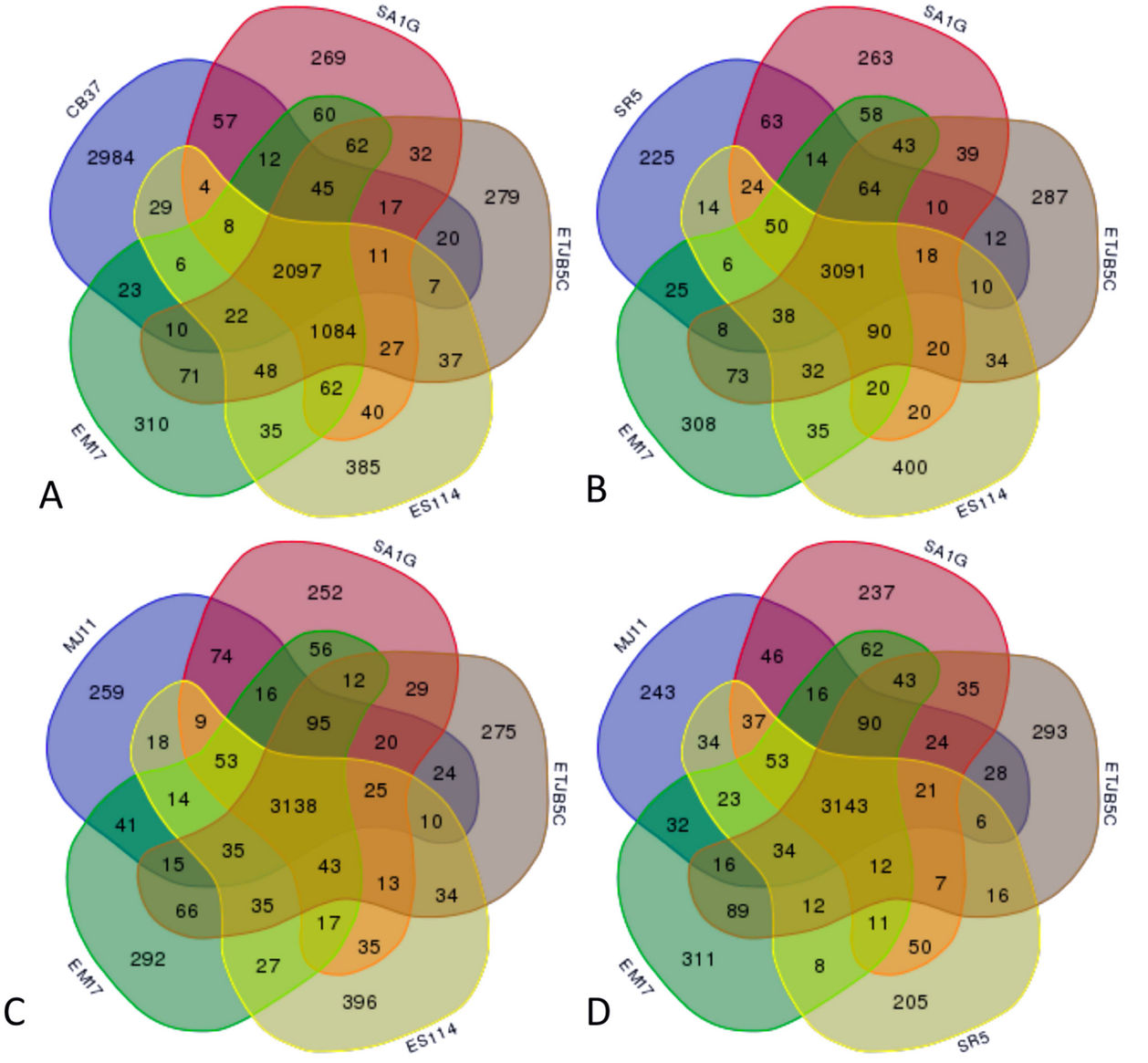


Fig. 1. – Venn diagrams from the Bioinformatics and Evolutionary Genomics Tool depict shared gene content graphically for up to 5 genomes. A. Venn diagram depicting our three sequenced strains of symbiotically competent *V. fischeri* (SA1G, EM17, ETJB5C), a model organism (ES114), and the free-living *V. cf. campbellii* CB37. Here, we compare our free-living strain, CB37, to our symbiotic strains. B. Venn diagram depicting symbiotically competent *V. fischeri* that colonize squid hosts, including our three strains (SA1G, EM17, ETJB5C), a model organism (ES114), and another previously published squid symbiont (SR5). Here, we compare our symbiotic strains to other squid symbiotic strains. C. Venn diagram depicting four strains of symbiotically competent *V. fischeri* with squid hosts (SA1G, EM17, ETJB5C, ES114) and a fish host (MJ11). D. Venn diagram depicting four strains of symbiotically competent *V. fischeri* with squid hosts (SA1G, EM17, ETJB5C, SR5) and a fish host (MJ11). Note that the core genomes when including the fish symbionts (panels C, D) are actually larger (3138 vs. 3143) than the core genome for just the squid symbionts (panel B; 3091), indicating that the fish symbiont is not the most divergent of the symbiotic strains.

event in SR5 to 51 found in EM17 (Supplemental Table VI).

In pairwise comparisons of genetic diversity among the symbiotic *V. fischeri* strains, the greatest difference was seen between SA1G and ES114, with their orthologous genes differing at approximately 3.8 % of nucleotide sites (Table III). The most similar pairing was between SA1G and MJ11, where approximately 1.8 % of their orthologs differed in nucleotide sequence (Table III).

Functional groups analysis

The number of genes assigned to different functional groups varies between symbiotic genomes and the free-living CB37 (RAST analysis; Table IV). Annotations of all specific genes recovered from this RAST analysis are given in Supplementary Table IV.

GC analysis

Distributions of GC proportions by gene for all four genomes sequenced in this study were not normal but were skewed towards ORFs with lower GC content than the genome mean (ETJB5C, $D = 0.065$, $p < 0.01$; SA1G, $D = 0.062$, $p < 0.01$; EM17, $D = 0.060$, $p < 0.01$; CB37, $D = 0.088$, $p < 0.01$; Table V). A high frequency of genes at the edges of the distribution of GC proportions (more than 2 SDs from the mean) for each genome were spatially clustered; that is, they were proximate in the genome (Table V, Supplementary Table V).

Positive selection analysis

A total of 38 genes show evidence in at least one pairwise-comparison of positive selection with ratios greater than 1.0: twenty two in EM17, ten in ES114, four in ETJB5C, and one each in MJ11 and SA1G (Table VI).

Phylogenetic analysis

The maximum likelihood approach of PhyML was used to create a phylogeny of our *V. fischeri* strains. We collected 1,870 orthologs from our three symbiotically competent *V. fischeri* strains (EM17, ETJB5C, SA1G), one free-living strain used as an outgroup (CB37), and three other symbiotic *V. fischeri* strains (ES114 (Ruby *et al*. 2005), MJ11 (Mandel *et al*. 2009), SR5 (Gyllborg *et al*. 2012). These orthologs were aligned and concatenated at the protein level to generate multiple sequence alignments. The best-supported tree identified two clades, one displaying EM17 as sister to ETJB5C and ES114, and the other with SR5 as sister to SA1G and MJ11 (Fig. 2), with *V. cf. campbellii* CB37 as the outgroup. High bootstrap values were obtained for all tree nodes.

Table IV. – Variance in genes comprising functional groups of newly sequenced genomes of *Vibrio* species and one model organism *V. fischeri* ES114.

Subsystem feature counts	CB37	ETJB5C	EM17	SA1G	ES114
Amino Acids and Derivatives	451	324	328	329	329
Carbohydrates	576	375	371	414	414
Cell Division and Cell Cycle	33	31	35	33	33
Cell Wall and Capsule	183	205	176	191	191
Cofactors, vitamins, prosthetic groups, pigments	254	202	202	205	205
DNA metabolism	149	134	151	132	132
Dormancy and Sporulation	7	7	4	4	4
Fatty Acids, Lipids, and Isoprenoids	131	118	107	124	124
Iron Acquisition and Metabolism	83	50	55	72	72
Membrane transport	227	194	200	199	199
Metabolism of aromatic compounds	14	4	4	4	4
Miscellaneous	193	175	170	184	184
Motility and chemotaxis	164	107	108	101	101
Nitrogen metabolism	50	38	38	39	39
Nucleosides and nucleotides	110	95	99	94	94
Phages, prophages, transposable elements, plasmids	9	7	19	9	9
Phosphorus metabolism	58	37	38	36	36
Potassium metabolism	58	53	51	52	52
Protein metabolism	270	232	238	241	241
Regulation and cell signaling	119	99	92	93	93
Respiration	185	131	129	130	130
RNA metabolism	220	141	142	141	141
Secondary metabolism	0	0	0	0	0
Stress response	228	185	184	188	188
Sulfur metabolism	30	32	28	29	29
Virulence, disease, and defense	106	76	75	73	73
Total open reading frames	3908	3052	3044	3117	3117

Table V. – Mean GC content within genomes of four *Vibrio* strains sequenced in this study. Distributions were skewed toward more open reading frames (ORFs) with low GC content (those with GC proportions farther than 2 standard deviations (SDs) from the mean). Clusters, defined as more than 3 ORFs in proximity, with high or low GC content are provided in Supplementary Table V.

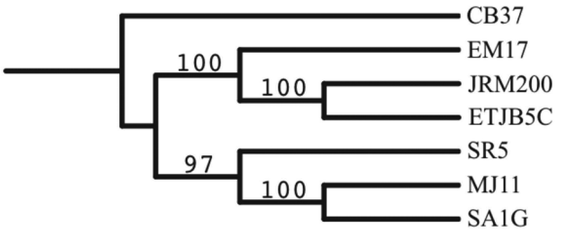
Strain	Mean ORF length (bp)	Mean GC proportion	Total ORFs	MAX GC proportion	MIN GC proportion	SD GC proportion	ORFs GC content above mean	ORFs GC content below mean	Number of ORFs in clusters	Proportion of ORFs in clusters
ETJB5C	952	0.3872	3864	0.5014	0.1795	0.0349	40	156	77	0.3929
SA1G	968	0.3873	3885	0.4987	0.2027	0.0352	42	140	31	0.1703
EM17	953	0.3883	3946	0.5014	0.2068	0.0353	48	146	32	0.1649
CB37	934	0.4535	5340	0.5464	0.2650	0.0318	17	250	78	0.2921

Table VI. – Positively-selected genes identified through pairwise comparative analyses of symbiotic strains.

Strain ID	Compared to:	Gene Annotation	Gene ID	Known or putative function	Reference
ES114	ETJB5C	Hypothetical protein	VF_A0138	Unknown	–
	MJ11	FhuC ferric hydroxamate uptake	VF_A0158	Iron transport	–
	ETJB5C	Hypothetical protein	VF_A0755	Unknown	–
	SA1G	RplP 50S ribosomal subunit protein L16	VF_0243	Translation	–
	SA1G	Crp/Fnr family transcriptional regulator	VF_0318	Environmental stress response	(Soto & Nishiguchi 2014)
	EM17	ProW glycine betaine/proline ABC transporter permease	VF_0786	Osmoprotection	(Chavez-Dozal *et al.* 2014)
	SA1G	Hypothetical protein	VF_1745	Unknown	–
	ETJB5C	Hypothetical protein	VF_1916	Unknown	–
	ETJB5C	AtpI ATP synthase subunit I	VF_2571	Respiration	–
	SA1G	PanB pantothenate hydroxymethyltransferase	VF_2169	Pantothenate biosynthesis (FA, TCA metabolism)	(Jones *et al.* 1993)
ETJB5C	MJ11	ProtC transcriptional regulatory protein	277	Possible antibiotic resistance	(Wietzorrek & Bibb 1997)
	EM17	PriA primosome assembly protein	665	Transcription	–
	ES114	Hypothetical protein	2268	Unknown	–
	EM17; ES114	Phage shock protein	3285	Possible extracytoplasmic stress response	(Maxson & Darwin 2004)
EM17	MJ11	Glutathione S-transferase	190	Detoxification	(Vuilleumier 1997)
	ES114	RpoZ DNA-directed RNA polymerase subunit omega	691	Transcription	–
	ES114	ProQ solute/DNA competence effector	933	Osmoprotectant regulation	(Browne-Silva & Nishiguchi 2008)
	ES114	Hypothetical protein	1289	Unknown	–
	SA1G	Hypothetical protein	1433	Unknown	–
	ETJB5C	Hypothetical protein	1936	Unknown	–
	ETJB5C	Surface protein	1992	Unknown	–
	ES114	Hypothetical protein/possible peptidase	2476	Unknown	–
	ES114; ETJB5C	RpsU 30S ribosomal protein S21	2576	Translation	–
	ETJB5C	PilP pili assembly protein	2620	Conjugation/twitching motility	–
	ES114; ETJB5C	tRNA dihydrouridine synthase B	2709	Translation	–
	ETJB5C	RplU 50S ribosomal protein L21	2794	Translation	–
	ES114	RaiA stationary phase translation inhibitor and ribosome stability factor	3022	Translation	–
	ETJB5C	Type VI secretion system lysozyme-related protein	3088	Possible competition role	(Soto *et al.* 2014)
	ETJB5C	Putative penicillin-binding protein 1C	3124	Antibiotic resistance	–
	ES114	RpsF 30S ribosomal protein S6	3179	Translation	–
	ETJB5C	Hypothetical protein	3250	Unknown	–
	ETJB5C	Hypothetical protein	3804	Unknown	–
	SA1G	RhlE RNA helicase	1433	Transcription	–

Table VI. – Continued.

Strain ID	Compared to:	Gene Annotation	Gene ID	Known or putative function	Reference
	MJ11	Putative lipoprotein	1644	Membrane function/ environmental sensing	–
	ETJB5C	DksA RNA polymerase binding protein	2388	Transcription	–
	ETJB5C	MshO mannose-sensitive hemagglutinin type IV pilus assembly	2722	Host colonization	–
SA1G	MJ11	PilZ Type IV pilus assembly protein	3870	Motility/biofilm formation	(Mattick 2002)
MJ11	ETJB5C	Beta-lactamase	4686	Antibiotic resistance	(Ambler 1980)



Key: CB37 is a free-living *Vibrio harveyi* collected in Australia; EM17 is a *Vibrio fischeri* isolated from *Euprymna morsei* from Japan; JRM200 is a *V. fischeri* isolated from *E. scolopes*, Hawaii; ETJB5C is a *V. fischeri* isolated from *E. tasmanica*, Australia; SR5 is a *V. fischeri* isolated from *Sepiola robusta* from the Mediterranean Sea; MJ11 is a *V. fisheri* fish symbiont isolated from *Monocentris japonica*, Japan; and, SA1G is a *V. fischeri* isolated from *S. affinis* from the Mediterranean Sea.

Fig. 2. – Maximum-likelihood bootstrap consensus tree constructed using PhyML 3.0 and showing relationships among *V. fischeri* strains sequenced in this study. The tree was generated using concatenated amino acid sequences from 1870 orthologous genes shared by all strains. We adopted the LG amino acid replacement matrix model (Le & Gascuel 2008) and utilized the SPR option, which provides the slowest but most accurate tree-topology search. Bootstrap values for nodes were generated using 100 replications.

DISCUSSION

Overview

The plethora of genes divergent among our three symbiotic strains potentially reflect different environmentally-selective regimes and different hosts as well as several divergent between all symbiotic and obligately free-living vibrios (Table III). Rapid Annotation using Subsystem Technology (RAST) uses its own algorithms to discover open reading frames and bin them into functional groups/subsystems (Overbeek *et al*. 2014); different strains have different numbers of genes in each subsystem, which may be indicative of varying environments. Genes under positive selection were also detected and may encompass both host- and free-living effects (Table VI). Within the three symbiotic strains examined, genomes are relatively conserved with evidence of strong purifying selection and few duplication events. Interestingly, a reduced genome size, compared to that of other vibrios we used as reference (mean 3898 vs. 5173 genes), may be indicative of tradeoffs to allow rapid reproduction within host light organs, despite the fact that *V. fischeri* must cycle through a free-living stage. This is common in vertically-transmitted, obligate mutualisms, where bacterial symbionts are streamlined to the specific capabilities that are encompassed during symbiosis (Moran & Mira 2001, Moran 2002, Toh *et al*. 2006, McCutcheon & Moran 2012).

Symbiotic strains demonstrate substantial variation in their accessory genomes presumably due to the necessity of adapting to both the specific host species and unique abiotic environment faced by each *V. fischeri* strain (Fig. 1). Some of these may be acquired via horizontal gene transfer (HGT) as suggested by GC signatures that deviate significantly from mean genome proportions (Table V). The proximity or clustering of ORFs with similar GC frequencies that deviate > 2 SDs from the mean of the genome illustrates that genes with lower than average GC content are not randomly distributed throughout the genome. These blocks of ORFs may be of interest to assess HGT, which may be indicated by either abnormally high or low GC content (Garcia-Vallvé *et al*. 2000, Marcus *et al*. 2000), and which often occurs in the form of pathogenicity islands where several ORFs of an operon are transferred in one event. However, other factors may influence GC content (Hildebrand *et al*. 2010, Hayek 2013), and a further analysis of these blocks is warranted and should be addressed in a separate, more in-depth analysis. Other forces beyond vertical inheritance are also indicated by the phylogenetic analysis, which lacks a biogeographic signature (Fig. 2). Each symbiont's genome contains unique elements that may be shaped by selection from both host and environment; details are summarized in the sections below.

Genome level summary

Our three symbiotic strains of *V. fischeri* exhibited 3,186 orthologous genes (approx. 81.7 %), which comprise the core genome. Our strains combined with two other published squid symbionts had a core genome of 3,091 orthologs; when fish symbiont MJ11 is added to the analysis, there are 3018 (approx. 78 %) shared orthologs (Fig. 1). In comparison, four strains of *V. vulnificus* shared 3,459 genes (Gulig *et al*. 2010), possibly suggesting that pathogenicity in vibrio strains requires a slightly larger suite of conserved genes than does mutualism or

that adaptation to different host squids caused more divergence in our mutualistic genomes.

The accessory genomes uncovered in this study, which are by definition everything exclusive of the core genome, contain an average 833 genes, with each strain possessing an average of 226 unique genes; the remainder were paralogs. The strain-specific genes have no close homologs (> 60 % similarity over 50 % of length) in any of the other five *V. fischeri* strains we examined. Accessory genomes have been hypothesized to be reflective of environmental and host heterogeneity and to equip each strain with the suite of genes most adaptive to its unique ecological niche (Read & Ussery 2006, Mira *et al*. 2010). Strain-specific genes are postulated to arise either from gene duplication, leading to divergence and evolution of new or altered functions, or from lateral gene transfer, in which novel genes are appropriated from other organisms (Zhang 2003, Gevers *et al*. 2004, Treangen *et al*. 2009). However, a more recent analysis found that gene duplication may have a greater effect on gene dosage and less effect on gene neofunctionalization for shaping bacterial genomes (Treangen & Rocha 2011). The strain-specific genes identified through this study are keys to deciphering the relative significance of environmental and host-driven parameters in future studies of *V. fischeri* evolution.

In contrast to findings of significant gene duplication and lineage-specific expansion of protein families within Vibrionaceae (Gu *et al*. 2009), we saw little evidence of widespread gene duplication within symbiotic *V. fischeri* strains. *Vibrio fischeri* EM17 had the most paralogs contained in both its core and accessory genomes (260), while *V. fischeri* SR5 had the fewest (171). The average number of paralogs was 215, representing 5.6 % of the average genome size. Some duplicated genes within each symbiotic genome may have adaptive significance distributed between host and environmental effects, e.g., quadruplets of anaerobic glycerol subunits in SA1G shown to be upregulated during symbiosis (Wier *et al*. 2010) and duplicates of cold shock proteins in MJ11 possibly retained because of environmental effects (Supplementary Table VI).

The proportion of open reading frames associated with different subsystems as assigned during RAST analysis further demonstrates the evolutionary divergence between free-living and symbiotic strains. For example, the free-living *V. cf. campbellii* CB37 has a much larger genome, including many more coding regions associated with nutrient and energy acquisition (Table IV). The symbiotic genomes, in contrast, are streamlined and fairly conserved among each other. In addition, there are fewer elements associated with virulence, disease, and defense in the symbiotic genomes. This analysis showed conservation among the symbiotic genomes in broad functional categories, though some differences are apparent. "Iron acquisition and metabolism" may be limiting in SA1G and ES114 in comparison to ETJB5C and EM17 (Table IV). For example, the former two strains have genes for paraquat-inducible protein A (heme and hemin uptake), while the latter strains lack this gene (Supplementary Table IV). Both SA1G and ES114 have 17 and 22 more genes for "iron acquisition and metabolism" respectively, than EM17, and 22 and 27 more than ETJB5C (Supplementary Table IV). In contrast, the free-living *Vibrio* CB 37 had a total of 83 genes in that subcategory, suggesting that strain experiences the highest selection pressure to sequester a rare resource.

To highlight current levels of natural selection operating on symbiotic *V. fischeri* strains, we calculated ratios of non-synonymous to synonymous (K_a/K_s) substitutions among all possible pairwise comparisons of orthologous proteins. Positive selection was indicated for twenty-two genes in EM17, ten in ES114, four in ETJB5C, and one each in MJ11 and SA1G (Table VI). However, out of a total 19,762 pairwise comparisons, the vast majority of these exhibit very low K_a/K_s ratios, suggesting strong purifying selection occurring on most of the orthologous proteins. Many of the genes exhibiting evidence of positive selection are hypothetical proteins, with no known function. However, the positively-selected genes for which annotations are available include some that are likely influential in adaptation to both host and environment, including mannose-sensitive hemagglutinin *mshO* in *V. fischeri* EM17, expressed during host infection and colonization, and, in *V. fischeri* SA1G, type IV pilus assembly gene *pilZ*, important for motility and biofilm formation (Mattick 2002); see Table VI).

Comparisons with other *Vibrio* species

By comparing our sequence database with genomic sequences available on GenBank of *Vibrio fischeri* and six other vibrios (Table II), we were able to identify genes that are strongly conserved in the symbiotic strains but highly divergent or missing in free-living or pathogenic strains. These genes potentially represent core "symbiotic" genes that are indispensable for host colonization and are inviting candidates for mutational analyses to decipher specific functions that enable *V. fischeri*'s beneficial associations with eukaryotic hosts. Some of these conserved genes include sequences from MJ11, the fish symbiont, while others only include the squid symbionts and may be specific to this host type.

Among the set of conserved orthologs that include MJ11 sequences is *asc1B*, which encodes arylsulfatase regulator and has been shown to be important in other bacterial colonization events (Cheng *et al*. 1992, Morgan *et al*. 2004). Genes in which the MJ11 sequence varies by just one codon include *ntrC*, a gene encoding nitrogen regulatory response regulator/sigma 54 interaction protein that in *V. vulnificus* is involved in membrane saccharide synthesis, biofilm formation, and possibly carbohydrate metabolism (Kim *et al*. 2007) and in *V. fischeri*

is somehow involved in colonization (Hussa *et al*. 2007); and *mshJ*, part of the mannose-sensitive hemagglutinen type IV pilus operon. These genes have functions in the colonization and eventual formation of biofilm, which is crucial for the vibrio community that is established inside the squid light organ (Yip *et al*. 2005, Visick *et al*. 2007, Browne-Silva & Nishiguchi 2008, Geszvain & Visick 2008, Ariyakumar & Nishiguchi 2009, Yildiz & Visick 2009, Chavez-Dozal *et al*. 2012).

Finally, genes conserved within squid symbionts but more divergent in MJ11 include *aepA* encoding an exoenzyme regulatory protein with a putative hydrolase function (Murata *et al*. 1994) and a NADH-dependent flavin oxireductase gene involved in producing the substrate for luciferase (Duane & Hastings 1975). In the phytopathogen *Erwinia carotovora*, *aepA* is necessary for host infection and is up-regulated in response to quorum-sensing signals. Flavin oxireductase, which is instrumental for bioluminescence, has significant sequence divergence from the other *Vibrio* symbionts and is particularly divergent in SA1G, with otherwise close sequence similarity to MJ11. Presumably there are functional attributes of bioluminescence shared between SA1G and MJ11 but distinct from the other symbiotic strains. Whether these attributes relate to an ecological component common between SA1G and MJ11 or is phylogenetic convergence is not presently known.

Phylogenetic analysis

Phylogenetic analysis of the six symbiotic *V. fischeri* strains, using CB37 as the outgroup, revealed a pattern inconsistent with geography, host, or environmental parameters alone and suggests a complex evolutionary history within *V. fischeri* (Fig. 2). One clade consisted of EM17 as sister to ETJB5C and ES114, while a second clade contained SR5 as sister to SA1G and MJ11. These relationships do not have strictly biogeographical signatures; for example, both EM17 and MJ11 are Sea of Japan isolates, yet fall into separate clades. Similarly, the Mediterranean squid symbiotic strain SA1G is more closely related to MJ11 than it is to the other Mediterranean strain SR5. Presumably, different ecological constraints have imposed isolating barriers between *V. fischeri* populations geographically close to one another, despite their hosts sharing multiple species of *Vibrio* bacteria (Fidopiastis *et al*. 1998, Nishiguchi 2000, Zamborsky & Nishiguchi 2011).

Certainly, more extensive taxon sampling might help elucidate evolutionary relationships among symbiotic *V. fischeri* strains, but what our analysis suggests is that divergence of orthologous proteins follows neither clear biogeographical routes nor obvious environmental factors. Instead, evolution of this group of bacteria may operate in a fragmented manner, similar to what has been found in other groups of closely related bacteria, where evolutionary independence of orthologous genes contributes to patchy retention of genes acquired through homologous recombination with proximal populations of *V. fischeri* (Retchless & Lawrence 2010).

CONCLUSION

A selective balance presumably exists between environmental and biogeographical factors that shape the evolution of *V. fischeri* symbiotic strains, likely creating a selection mosaic dependent upon specific interactions between the bacteria, their hosts, and heterogenous environments (Fierer & Jackson 2006, Horner-Devine *et al*. 2004, Yannarell & Triplett 2005). Other researchers have demonstrated similar findings when examining microbial distributions and evolutionary relationships. For example, bacterial communities are driven primarily by environmental heterogeneity rather than geographic distance (Horner-Devine *et al*. 2004); diversity in soil bacterial communities is primarily controlled by edaphic factors (Fierer & Jackson 2006); and, bacterial community composition in shallow lake systems is strongly influenced by local environmental factors (Yannarell & Triplett 2005). The identification of genes that are unique to each strain used in our study, as well as those that exhibit positive selection signatures, is the first step toward disentangling the respective roles that the divergent environments within and outside the host have had on *V. fischeri* evolution.

However, the process of teasing apart environmental and host effects is complicated by the fact that many genes have multiple functions or are upregulated under numerous scenarios. For example, EM17 3088, a Type VI secretion system lysozyme-related protein, could potentially be used against competitors during host colonization (Brooks *et al*. 2013) or against predators in the water column (Pernthaler 2005). In the legume-rhizobia symbiosis, fully one-third of known symbiosis genes are involved in multiple pathways connected to cell metabolism, transcription, signal transduction, and protein modification and regulation (Tian *et al*. 2012). A similar cross-functionalization of symbiotic genes is likely to be valid in the squid-*Vibrio* mutualism.

In summary, *Vibrio fischeri* evolution is likely a patchwork of host- and abiotic environment-driven adaptation. Future work needs to employ comparative methods sampling more strains that differ in only one variable (host, temperature, salinity) but targeting one or a few of the genes/genetic elements we have identified here. While genomic and transcriptional studies have been extremely informative for this symbiosis complex thus far, the proposed strategy will maximize sequencing efficiency and provide more comparative power than previously available.

ACKNOWLEDGEMENTS. – Data are archived in GenBank as BioProject IDs PRJNA212805 (CB37); PRJNA212806 (EM17);

PRJNA212810 (SA1G); and PRJNA212811 (ETJB5C)). We would like to thank NMSU's 454 sequencing staff for help with this project, in particular Dr A Tchourbanov for his help with genome assemblies and Dr A Chavez-Dozal for the DNA preparations. We thank Dr D Houstan for comments on an earlier draft. This research was funded by grants to MKN from the National Institutes of Health NIAID 1SC1AI081659-01 and NIAID ARRA 3SC1AI081659-02S1, and National Science Foundation IOS-074449.

REFERENCES

Ambler RP 1980. The structure of beta-lactamases. *Philos Trans R Soc Lond B Biol Sci* 289(1036): 321-331.

Ariyakumar DS, Nishiguchi MK 2009. Characterization of two host-specific genes, mannose-sensitive hemagglutinin (*mshA*) and uridyl phosphate dehydrogenase (UDPDH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. *Fems Microbiol Lett* 299(1): 65-73.

Bartlett DH, Valle G, Lauro FM, Vezzi A, Simonato F, Eloe E, Vitulo N, Stratton TK, D'angelo M, Ferriera S, Johnson J, Kravitz S, Beeson K, Sutton G, Rogers Y, Friedman R, Frazier M, Venter JC 2006. *Photobacterium profundum* 3TCK, whole genome shotgun sequencing project. J Craig Venter Institute, Rockville, MD 20850, USA.

Bassler B, Clifton SW, Fulton L, Delehaunty K, Fronick C, Harrison M, Markivic C, Fulton R, Tin-Wollam AM, Shah N, Pepin K, Nash W, Thiruvilangam P, Bhonagiri V, Waters C, Tu KC Irgon J, Wilson RK 2007. The *Vibrio harveyi* Genome Sequencing Project. Genetics, Genome Sequencing Center, 4444 Forest Park Parkway, St. Louis, MO 63108, USA.

Boettcher KJ, Ruby EG, McFall-Ngai MJ 1996. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *J Comp Physiol A Sens Neural Behav Physiol* 179(1): 65-73.

Brooks TM, Unterweger D, Bachmann V, Kostiuk B, Pukatzki S 2013. Lytic activity of the *Vibrio cholerae* type VI secretion toxin VgrG-3 is inhibited by the antitoxin TsaB. *J Biol Chem* 288(11): 7618-7625.

Browne-Silva J, Nishiguchi M 2008. Gene sequences of the pil operon reveal relationships between symbiotic strains of *Vibrio fischeri*. *Int J Syst Evol Microbiol* 58(6): 1292-1299.

Chavez-Dozal A, Hogan D, Gorman C, Quintanal-Villalonga A, Nishiguchi MK 2012. Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. *FEMS Microbiol Ecol* 81(3): 562-573.

Chavez-Dozal AA, Gorman C, Lostroh CP, Nishiguchi MK 2014. Gene-swapping mediates host specificity among symbiotic bacteria in a beneficial symbiosis. *PLoS ONE* 9(7): e101691.

Chavez-Dozal A, Nishiguchi MK 2011. Variation in biofilm formation among symbiotic and free-living strains of *Vibrio fischeri*. *J Basic Microbiol* 51(5): 452-458.

Chen F, Mackey AJ, Stoeckert CJ, Jr Roos DS 2006. OrthoMCL-DB: querying a comprehensive multi-species collection of ortholog groups. *Nucleic Acids Res* 34(Database issue): D363-368.

Cheng Q, Hwa V, Salyers AA 1992. A locus that contributes to colonization of the intestinal tract by *Bacteroides thetaiotaomicron* contains a single regulatory gene (*chuR*) that links 2 polysaccharide utilization pathways. *J Bacteriol* 174(22): 7185-7193.

Chun CK, Troll JV, Koroleva I, Brown B, Manzella L, Snir E, Almabrazi H, Scheetz TE, Bonaldo MD, Casavant TL, Soares MB, Ruby EG, McFall-Ngai MJ 2008. Effects of colonization, luminescence, and autoinducer on host transcription during development of the squid-*Vibrio* association. *Proc Nat Acad Sci USA* 105(32): 11323-11328.

Coleman ML, Chisholm SW 2010. Ecosystem-specific selection pressures revealed through comparative population genomics. *Proc Nat Acad Sci USA* 107(43): 18634-18639.

Davidson SK, Koropatnick TA, Kossmehl R, Sycuro L, McFall-Ngai MJ 2004. NO means 'yes' in the squid-*Vibrio* symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. *Cell Microbiol* 6(12): 1139-1151.

Dong TG, Ho BT, Yoder-Himes DR, Mekalanos JJ 2013. Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. *PNAS* 110(7): 2623-2628.

Duane W, Hastings JW 1975. Flavin mononucleotide reductase of luminous bacteria. *Mol Cell Biochem* 6(1): 53-64.

Eberhard A 1972. Inhibition and activation of bacterial luciferase synthesis. *J Bacteriol* 109(3): 1101-1105.

Edgar RC 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acid Res* 32(5): 1792-1797.

Ellison CE, Hall C, Kowbel D, Welch J, Brem RB, Glass NL, Taylor JW 2011. Population genomics and local adaptation in wild isolates of a model microbial eukaryote. *PNAS USA* 108(7): 2831-2836.

Fidopiastis PM, von Boletzky S, Ruby EG 1998. A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. *J Bacteriol* 180(1): 59-64.

Fierer N, Jackson RB 2006. The diversity and biogeography of soil bacterial communities. *PNAS USA* 103(3): 626-631.

Fuqua WC, Winans SC, Greenberg EP 1994. Quorum sensing in bacteria - the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* 176(2): 269-275.

Garcia-Vallvé S, Romeu A, Palau J 2000. Horizontal gene transfer in bacterial and archaeal complete genomes. *Genome Res* 10(11): 1719-1725.

Geszvain K, Visick KL 2008. The hybrid sensor kinase RscS integrates positive and negative signals to modulate biofilm formation in *Vibrio fischeri*. *J Bacteriol* 190(13): 4437-4446.

Gevers D Vandepoele K Simillion C Van de Peer Y 2004. Gene duplication and biased functional retention of paralogs in bacterial genomes. *Trends Microbiol* 12(4): 148-154.

Gilad Y, Pritchard JK, Thornton K 2009. Characterizing natural variation using next-generation sequencing technologies. *Trends Genet* 25(10): 463-471.

Graf J, Ruby EG 1998. Host-derived amino acids support the proliferation of symbiotic bacteria. *PNAS USA* 95(4): 1818-1822.

Gu JY, Neary J, Cai H, Moshfeghian A, Rodriguez SA, Lilburn TG, Wang YF 2009. Genomic and systems evolution in Vibrionaceae species. *BMC Genomics* 10(Suppl 1): S11-24.

Guerrero-Ferreira RC, Nishiguchi MK 2010. Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches. *Environ Microbiol Rep* 2(4): 514-523.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59(3): 307-321.

Gulig PA, de Crecy-Lagard V, Wright AC, Walts B, Telonis-Scott M, McIntyre LM 2010. SOLiD sequencing of four *Vibrio vulnificus* genomes enables comparative genomic analysis and identification of candidate clade-specific virulence genes. *BMC Genomics* 11: 512.

Gyllborg MC, Sahl JW, Cronin DC 3rd, Rasko, DA Mandel MJ 2012. Draft genome sequence of *Vibrio fischeri* SR5, a strain isolated from the light organ of the Mediterranean squid *Sepiola robusta*. *J Bacteriol* 194(6): 1639.

Haardt M, Bremer E 1996. Use of phoA and lacZ fusions to study the membrane topology of ProW, a component of the osmoregulated ProU transport system of *Escherichia coli*. *J Bacteriol* 178(18): 5370-5381.

Hayek N 2013. Lateral transfer and GC content of bacterial resistance genes. *Front Microbiol* 4: 41.

Hildebrand F, Meyer A, Eyre-Walker A 2010. Evidence of selection upon genomic GC-content in bacteria. *PLoS Genet* 6(9): e1001107.

Horner-Devine MC, Lage M, Hughes JB, Bohannan BJ 2004. A taxa-area relationship for bacteria. *Nature* 432(7018): 750-753.

Hudson ME 2008. Sequencing breakthroughs for genomic ecology and evolutionary biology. *Mol Ecol Resour* 8(1): 3-17.

Hussa EA, O'Shea, TM Darnell, CL Ruby, EG Visick KL 2007. Two-component response regulators of *Vibrio fischeri*: identification, mutagenesis, and characterization. *J Bacteriol* 189(16): 5825-5838.

Jones BW, Nishiguchi MK 2004. Counterillumination in the hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar Biol* 144(6): 1151-1155.

Jones BW, Lopez JE, Huttenburg J, Nishiguchi MK 2006. Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol Ecol* 15(14): 4317-4329.

Jones BW, Nishiguchi MK 2006. Differentially expressed genes reveal adaptations between free-living and symbiotic niches of *Vibrio fischeri* in a fully established mutualism. *Can J Microbiol* 52(12): 1218-1227.

Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK 2007. Spatial and temporal distribution of the Vibrionaceae in coastal waters of Hawaii, Australia, and France. *Microb Ecol* 54(2): 314-323.

Jones CE, Brook JM, Buck D, Abell C, Smith AG 1993. Cloning and sequencing of the *Escherichia coli* panB gene, which encodes ketopantoate hydroxymethyltransferase, and overexpression of the enzyme. *J Bacteriol* 175(7): 2125-2130.

Keymer DP, Miller MC, Schoolnik GK, Boehm AB 2007. Genomic and phenotypic diversity of coastal *Vibrio cholerae* strains is linked to environmental factors. *Appl Environ Microbiol* 73(11): 3705-3714.

Kim HS, Lee MA, Chun SJ, Park SJ, Lee KH 2007. Role of *ntrC* in biofilm formation via controlling expression of the gene encoding an ADP-glycero-manno-heptose-6-epimerase in the pathogenic bacterium, *Vibrio vulnificus*. *Mol Microbiol* 63(2): 559-574.

Le SQ Gascuel O 2008. An improved general amino acid replacement matrix. *Mol Biol Evol* 25(7): 1307-1320.

Lee KH, Ruby EG 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl Environ Microbiol* 60(5): 1565-1571.

Levasseur A, Pontarotti P 2011. The role of duplications in the evolution of genomes highlights the need for evolutionary-based approaches in comparative genomics. *Biol Direct* 6: 11.

Lin B, Wang Z, Malanoski AP, O'Grady EA, Wimpee CF, Vuddhakul V, Alves N Jr, Thompson FL, Gomez-Gil B Vora GJ 2010. Comparative genomic analyses identify the *Vibrio harveyi* genome sequenced strains BAA-1116 and HY01 as *Vibrio campbellii*. *Environ Microbiol Rep* 2(1): 81-89.

Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG 2009. A single regulatory gene is sufficient to alter bacterial host range. *Nature* 458(7235): 215-218.

Marcus SL, Brumell JH, Pfeifer CG, Finlay BB 2000. *Salmonella* pathogenicity islands: big virulence in small packages. *Microbes Infect* 2(2): 145-156.

Mardis ER 2008. The impact of next-generation sequencing technology on genetics. *Trends Genet* 24(3): 133-141.

Mattick JS 2002. Type IV pili and twitching motility. *Annu Rev Microbiol* 56: 289-314.

Maxson ME, Darwin AJ 2004. Identification of inducers of the *Yersinia enterocolitica* phage shock protein system and comparison to the regulation of the RpoE and Cpx extracytoplasmic stress responses. *J Bacteriol* 186(13): 4199-4208.

McCutcheon JP, Moran NA 2012. Extreme genome reduction in symbiotic bacteria. *Nat Rev Microbiol* 10(1): 13-26.

Medina M, Sachs JL 2010. Symbiont genomics, our new tangled bank. *Genomics* 95(3): 129-137.

Metzker ML 2010. Sequencing technologies - the next generation. *Nat Rev Genet* 11(1): 31-46.

Mira A, Martin-Cuadrado AB, D'Auria G, Rodriguez-Valera F 2010. The bacterial pan-genome: a new paradigm in microbiology. *Int Microbiol* 13(2): 45-57.

Moran NA, Mira A 2001. The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola*. *Genome Biol* 2(12): RESEARCH0054.

Moran NA 2002. Microbial minimalism: genome reduction in bacterial pathogens. *Cell* 108(5): 583-586.

Morgan E, Campbell JD, Rowe SC, Bispham J, Stevens MP, Bowen AJ, Barrow PA, Maskell DJ, Wallis TS 2004. Identification of host-specific colonization factors of *Salmonella enterica* serovar *Typhimurium*. *Mol Microbiol* 54(4): 994-1010.

Morris AR, Visick KL 2010. Control of biofilm formation and colonization in *Vibrio fischeri*: a role for partner switching? *Environ Microbiol* 12(8): 2051-2059.

Murata H, Chatterjee A, Liu Y, Chatterjee AK 1994. Regulation of the production of extracellular pectinase, cellulase, and protease in the soft rot bacterium *Erwinia carotovora* subsp. *carotovora*: evidence that *aepH* of *E. carotovora* subsp. *carotovora* 71 activates gene expression in *E. carotovora* subsp. *carotovora, E. carotovora* subsp. *atroseptica*, and *Escherichia coli*. *Appl Environ Microbiol* 60(9): 3150-3159.

Nadeau NJ, Jiggins CD 2010. A golden age for evolutionary genetics? Genomic studies of adaptation in natural populations. *Trends Genet* 26(11): 484-492.

Nealson KH 1977. Autoinduction of bacterial luciferase. Occurrence, mechanism and significance. *Arch Microbiol* 112(1): 73-79.

Nishiguchi MK, Ruby EG, McFall-Ngai MJ 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-vibrio symbioses. *Appl Environ Microbiol* 64(9): 3209-3213.

Nishiguchi MK 2000. Temperature affects species distribution in symbiotic populations of Vibrio spp. *Appl Environ Microbiol* 66(8): 3550-3555.

Nishiguchi MK 2002. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. *Microb Ecol* 44(1): 10-18.

Nishiguchi MK, DeVinney R, Hirsch AM, Riley M, Mansky L, Vendatum G 2008. Perspective: Evolution of Virulence: Deciphering the mechanisms between pathogenic and benign symbioses. *Vie Milieu* 58(2): 87-106.

Nyholm SV, McFall-Ngai MJ 2004. The winnowing: Establishing the squid-*Vibrio* symbiosis. *Nat Rev Microbiol* 2(8): 632-642.

Nyholm SV, Nishiguchi MK 2008. The evolutionary ecology of a sepiolid squid-*Vibrio* association: From cell to environment. *Vie Milieu* 58(2): 175-184.

Orr HA 2005. The genetic theory of adaptation: A brief history. *Nat Rev Genet* 6(2): 119-127.

Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S Parrello B, Shukla M 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* 42(D1): D206-D214.

Pernthaler J 2005. Predation on prokaryotes in the water column and its ecological implications. *Nat Rev Microbiol* 3(7): 537-546.

Purushe J, Fouts DE, Morrison M, White BA, Mackie RI, Coutinho PM, Henrissat B, Nelson KE 2010. Comparative genome analysis of *Prevotella ruminicola* and *Prevotella bryantii*: insights into their environmental niche. *Microb Ecol* 60(4): 721-729.

Read TD, Ussery DW 2006. Opening the pan-genomics box. *Curr Opin Microbiol* 9(5): 496-498.

Retchless AC, Lawrence JG 2010. Phylogenetic incongruence arising from fragmented speciation in enteric bacteria. *PNAS USA* 107(25): 11453-11458.

Roh H, Yun EJ, Lee S, Ko HJ, Kim S, Kim BY, Song H, Lim KI, Kim KH, Choi IG 2012. Genome sequence of *Vibrio* sp. strain EJY3, an agarolytic marine bacterium metabolizing 3,6-anhydro-L-galactose as a sole carbon source. *J Bacteriol* 194(10): 2773-2774.

Ruby EG, Lee KH 1998. The *Vibrio fischeri-Euprymna scolopes* light organ association: Current ecological paradigms. *Appl Environ Microbiol* 64(3): 805-812.

Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gunsalus R, Lostroh P, Lupp C McCann J, Millikan D, Schaefer A, Stabb E, Stevens A, Visick K, Whistler C, Greenberg EP 2005. Complete genome sequence of *Vibrio fischeri*: A symbiotic bacterium with pathogenic congeners. *PNAS USA* 102(8): 3004-3009.

Ruby EG 2008. Symbiotic conversations are revealed under genetic interrogation. *Nat Rev Microbiol* 6(10): 752-762.

Sachs JL, Simms EL 2006. Pathways to mutualism breakdown. *Trends Ecol Evol* 21(10): 585-592.

Sachs JL, Russell JE, Hollowell AC 2011. Evolutionary instability of symbiotic function in *Bradyrhizobium japonicum*. *PLoS ONE* 6(11): e26370.

Schuster BM, Perry LA, Cooper VS, Whistler CA 2010. Breaking the language barrier: experimental evolution of non-native *Vibrio fischeri* in squid tailors luminescence to the host. *Symbiosis* 51(1): 85-96.

Smith MN, Crane RA, Keates RA Wood JM 2004. Overexpression, purification, and characterization of ProQ, a posttranslational regulator for osmoregulatory transporter ProP of *Escherichia coli*. *Biochemistry* 43(41): 12979-12989.

Soto W, Gutierrez J, Remmenga MR, Nishiguchi MK 2009a. Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. *Microb Ecol* 57(1): 140-150.

Soto W, Lostroh CP, Nishiguchi MK 2009b. Physiological responses to stress in the Vibrionaceae: aquatic microorganisms frequently affiliated with hosts. Springer, New York.

Soto W, Punke EB, Nishiguchi MK 2012. Evolutionary perspectives in a mutualism of sepiolid squid and bioluminescent bacteria: combined usage of microbial experimental evolution and temporal population genetics. *Evolution* 66(5): 1308-1321.

Soto W, Rivera FM, Nishiguchi MK 2014. Ecological diversification of *Vibrio fischeri* serially passaged for 500 generations in novel squid host *Euprymna tasmanica*. *Microb Ecol* 67(3): 700-721.

Stapley J, Reger J, Feulner PG, Smadja C, Galindo J, Ekblom R, Bennison C, Ball AD, Beckerman AP, Slate J 2010. Adaptation genomics: the next generation. *Trends Ecol Evol* 25(12): 705-712.

Suzuki H, Lefebure T, Hubisz MJ, Bitar PP, Lang P, Siepel A, Stanhope MJ 2011. Comparative genomic analysis of the *Streptococcus dysgalactiae* species group: gene content, molecular adaptation, and promoter evolution. *Genome Biol Evol* 3(168-185.

Tian CF, Zhou YJ, Zhang YM, Li QQ, Zhang YZ, Li DF, Wang S, Wang J, Gilbert LB, Li YR 2012. Comparative genomics of rhizobia nodulating soybean suggests extensive recruitment of lineage-specific genes in adaptations. *PNAS* 109(22): 8629-8634.

Toh H, Weiss BL, Perkin SA, Yamashita A, Oshima K, Hattori M, Aksoy S 2006. Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. *Genome Res* 16(2): 149-156.

Treangen TJ, Abraham AL, Touchon M, Rocha EPC 2009. Genesis, effects and fates of repeats in prokaryotic genomes. *FEMS Microbiol Rev* 33(3): 539-571.

Treangen TJ, Rocha EP 2011. Horizontal transfer, not duplication, drives the expansion of protein families in prokaryotes. *PLoS Genet* 7(1): e1001284.

Visick KL, O'Shea TM, Klein AH, Geszvain K, Wolfe AJ 2007. The sugar phosphotransferase system of *Vibrio fischeri* inhibits both motility and bioluminescence. *J Bacteriol* 189(6): 2571-2574.

Vuilleumier S 1997. Bacterial glutathione S-transferases: What are they good for? *J Bacteriol* 179(5): 1431-1441.

Wang YL, Dunn AK, Wilneff J, McFall-Ngai MJ, Spiro S Ruby EG 2010. *Vibrio fischeri* flavohaemoglobin protects against nitric oxide during initiation of the squid-*Vibrio* symbiosis. *Mol Microbiol* 78(4): 903-915.

Wier AM, Nyholm SV, Mandel MJ, Massengo-Tiasse RP, Schaefer AL, Koroleva I, Splinter-BonDurant S, Brown B, Manzella L, Snir E, Almabrazi H, Scheetz TE, Bonaldo MD, Casavant TL, Soares MB, Cronan JE, Reed JL, Ruby EG, McFall-Ngai MJ 2010. Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. *PNAS USA* 107(5): 2259-2264.

Wietzorrek A, Bibb M 1997. A novel family of proteins that regulates antibiotic production in streptomycetes appears to contain an *ompR*-like DNA-binding fold. *Mol Microbiol* 25(6): 1181-1184.

Wollenberg MS, Ruby EG 2009. Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* squid from two Oahu (Hawaii) populations. *Appl Environ Microbiol* 75(1): 193-202.

Yang Z 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Mol Biol Evol* 24(8): 1586-1591.

Yannarell AC, Triplett EW 2005. Geographic and environmental sources of variation in lake bacterial community composition. *Appl Environ Microbiol* 71(227-239.

Yildiz FH, Visick KL 2009. *Vibrio* biofilms: so much the same yet so different. *Trends Microbiol* 17(3): 109-118.

Yip ES, Grublesky BT, Hussa EA, Visick KL 2005. A novel, conserved cluster of genes promotes symbiotic colonization and sigma-dependent biofilm formation by *Vibrio fischeri*. *Mol Microbiol* 57(5): 1485-1498.

Zamborsky DJ, Nishiguchi MK 2011. Phylogeographical patterns among Mediterranean Sepiolid squids and their *Vibrio* symbionts: environment drives specificity among sympatric species. *Appl Environ Microbiol* 77(2): 642-649.

Zhang JZ 2003. Evolution by gene duplication: an update. *Trends Ecol Evol* 18(6): 292-298.

Zhou A, Chen YI, Zane GM, He Z, Hemme CL, Joachimiak MP, Baumohl JK, He Q, Fields MW, Arkin AP 2012. Functional characterization of Crp/Fnr-type global transcriptional regulators in *Desulfovibrio vulgaris* Hildenborough. *Appl Environ Microbiol* 78(4): 1168-1177.

Received on June 9, 2015
Accepted on August 24, 2015
Associate editor: A Chenuil

These supplementary tables are available on our website:

Supplementary Table I. – Genomic data summary and percent coverage of reference strains available on GenBank corresponding with newly sequenced genomes. NE = not examined.

Supplementary Table II. – Data concerning Roche 454 sequencing read quality.

Supplemental Table III. – List of core genes shared by symbiotic strains of *Vibrio fischeri*. The gene numbers are based on those for the V. fischeri MJ11 reference genome.

Supplementary Table IV. – Genetic elements identified in three symbiotic strains of *Vibrio fischeri* and one free-living *Vibrio* sp. sequenced in this study, in addition to one model organism, *V. fischeri* ES114 (Ruby *et al*. 2005). Annotations provided by RAST (Overbeek *et al*. 2014).

Supplementary Table V. – Patterns of GC content in Vibrio genomes sequenced in this study. Clusters of open reading frames (ORFs) that deviate from mean GC content are provided in blocks separated by spaces. Clusters are defined as 3 or more ORFs in proximity. High or low GC content is defined as GC proportions that fall more the 2 standard deviations from the mean for that genome.

Supplementary Table VI. – Gene duplication within symbiotic *Vibrio fischeri* and the non-symbiotic *V. cf. campbellii* strains. From the orthology analysis, amino acid sequences demonstrating closest-match sequence similarity to other genes within the sequence dataset are listed below. Lengths of the sequences are denoted by nucleotide number. Similarities to most closely-matched reference genomes are shown, as well as putative gene functions for these duplicated gene sequences.

Comparative analysis of quantitative methodologies for Vibrionaceae *biofilms*

Alba A. Chavez-Dozal, Neda Nourabadi, Martina Erken, Diane McDougald & Michele K. Nishiguchi

Folia Microbiologica
Official Journal of the Institute of Microbiology, Academy of Sciences of the Czech Republic and Czechoslavak Society for Microbiology

ISSN 0015-5632
Volume 61
Number 6

Folia Microbiol (2016) 61:449-453
DOI 10.1007/s12223-016-0456-9

Springer

Folia Microbiol (2016) 61:449–453
DOI 10.1007/s12223-016-0456-9

Comparative analysis of quantitative methodologies for *Vibrionaceae* biofilms

Alba A. Chavez-Dozal[1,2] · Neda Nourabadi[3] · Martina Erken[4] · Diane McDougald[5,6] · Michele K. Nishiguchi[3]

Received: 8 October 2015 / Accepted: 9 March 2016 / Published online: 24 March 2016

Abstract Multiple symbiotic and free-living *Vibrio* spp. grow as a form of microbial community known as a biofilm. In the laboratory, methods to quantify *Vibrio* biofilm mass include crystal violet staining, direct colony-forming unit (CFU) counting, dry biofilm cell mass measurement, and observation of development of wrinkled colonies. Another approach for bacterial biofilms also involves the use of tetrazolium (XTT) assays (used widely in studies of fungi) that are an appropriate measure of metabolic activity and vitality of cells within the biofilm matrix. This study systematically tested five techniques, among which the XTT assay and wrinkled colony measurement provided the most reproducible, accurate, and efficient methods for the quantitative estimation of *Vibrionaceae* biofilms.

✉ Michele K. Nishiguchi
nish@nmsu.edu

[1] Section of Infectious Diseases, New Mexico Veterans Healthcare System, Albuquerque, NM 87108, USA

[2] Division of Infectious Diseases, University of New Mexico Health Science Center, Albuquerque, NM 87131, USA

[3] Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA

[4] Centre for Marine Biofouling and Bio-Innovation, School of Biotechnology and Biomolecular Sciences, University of South Wales, Sydney, NSW 2052, Australia

[5] ithree Institute, Faculty of Science, University of Technology, Sydney, Broadway, Sydney, NSW 2007, Australia

[6] Singapore Centre on Environmental Life Sciences Engineering, School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore

Introduction

Biofilms are communities of microbes that are composed of cells attached to a surface and encapsulated in an extracellular matrix (composed primarily of polysaccharides, proteins, and DNA; Watnick and Kotler 2000; Yildiz and Visick 2008). Biofilms develop when cells transition from a planktonic (free-living) lifestyle to surface-attached complex multicellular communities (Watnick and Kotler 2000). These microscopic communities can form unique microbiomes that are common in nature, and can range from a healthy consortium of beneficial bacteria to those that can be the primary source of dangerous chronic diseases (Watnick and Kotler 2000; Costerton et al. 1999).

Biofilms formed by symbiotic bacteria in the family *Vibrionaceae* (pathogenic and mutualistic) have been studied for over 20 years, and diverse methodologies for studying *Vibrio* biofilms under laboratory conditions have been proposed by multiple research groups (Yildiz and Visick 2008). However, this area of research is in constant change and is still under development. For example, a recent methodology developed to measure *Vibrio* biofilm mass included examining cell viability and identification of common biofilm phenotypes (such as formation of wrinkled or rugose bacterial colonies; Ray et al. 2011), while another popular semiquantitative method (that has been used extensively for multiple bacterial biofilms) includes the use of crystal violet in a colorimetric assay to stain biofilms attached to a surface (O'Toole 2011). In the case of fungal biofilms (such as those formed by *Candida* and *Cryptococcus*), there is a commonly used colorimetric assay that accurately shows cellular viability within the biofilm through the metabolic use of formazan salts (Kuhn et al. 2003). Interestingly, this method is not routinely used in *Vibrio* (and other bacterial) biofilms for its quantitative capability or detection limits. All of these proposed methods

have been important tools to measure in vitro formation of biofilms. These procedures vary widely as to their time and cost requirements, and in variation reported in assay performance. An important element of these proposed methods is the necessity to accurately and reproducibly quantify viable cells in the biofilms as can be accomplished by a metabolism-based assay such as the XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide} reduction assay. Therefore, the goals of this current study were to test the efficacy of the XTT assay in *Vibrionaceae* biofilms as well as to make a comparative analysis of time, efficiency, and cost of different quantitative assays. Studies included the use of crystal violet staining, dry cell mass measurement, viable colony counting (direct enumeration of bacteria in biofilms), phenotype observation (wrinkled colony development), and the use of the XTT reduction assay.

Methods

Six wild-type *Vibrio* strains (biosafety level (BSL) 1) were selected for this study: *Vibrio rotiferianus* (Chowdhury et al. 2011), *Vibrio corallilyticus* (ATCC BAA450), *Vibrio parahaemolyticus* (ATCC 17802), two *Vibrio fischeri* strains isolated from *Euprymna* squid hosts: ES114 (*Euprymna scolopes* from Kaneohe Bay, O'ahu, HI, USA) and ETJB1H (*Euprymna tasmanica* from Jervis Bay, New South Wales, Australia), and one free-living (seawater) isolate (*V. fischeri* CB31 from Coogee Bay, New South Wales, Australia). We also selected mutant *V. fischeri* strains (from the ETJB1H isolate) that have been reported to be defective in biofilm formation (Ariyakumar and Nishiguchi 2009; Chavez-Dozal et al. 2012). Mutant strains had interruptions in genes responsible for (a) twitching motility and pilus assembly (Δ*pilT*, Δ*pilU*, Δ*mshA*; Ariyakumar and Nishiguchi 2009; Chavez-Dozal et al. 2012), (b) flagellum assembly and functionality (Δ*flgF*, Δ*motY*; Chavez-Dozal et al. 2012), and (c) stress responses such as heat shock (Δ*ibpA*) and magnesium-dependent induction (Δ*mifB*; Chavez-Dozal et al. 2012).

To evaluate biofilm formation, cultures were grown overnight at 28 °C and 250 rpm in Luria Bertani high-salt media (LBS; 10 g tryptone, 5 g yeast extract, 20 g sodium chloride, 50 mL 1 mol/L Tris pH 7.5, 3.75 mL 80 % glycerol, and 950 mL distilled water). Biofilm quantification was measured by five different methodologies, including crystal violet (CV) staining (O'Toole 2011), XTT assay (Pierce et al. 2010), dry cell mass measurement (Taff et al. 2012), colony counting (Merrit et al. 2005), and wrinkly colony development (Ray et al. 2011).

For the CV and XTT assays, all strains were subcultured and grown to a cell density of 1×10^8 colony-forming units (CFU)/mL. Aliquots of each *Vibrio* isolate (200 μL) were added to individual wells on a flat-bottom, polystyrene 96-well microtiter plate (Corning, Sigma-Aldrich CLS3628, St. Louis, MO) and incubated for 24 h under conditions previously described (Chavez-Dozal et al. 2012). After incubation, planktonic (those not forming biofilms) cells were removed by briskly shaking the plate and attached cells were washed three times with sterile media. For the CV assay, crystal violet (2 % aqueous solution) was added to each well and incubated at room temperature for 30 min. After incubation, CV was removed and the plate was washed five times with sterile media. CV was then quantified by solubilizing with 95 % ethanol, and optical density (A_{562}) readings were recorded at 562 nm for each biofilm in individual wells. For the XTT assay, planktonic cells were removed and plates washed as previously described (O'Toole 2011; Ariyakumar and Nishiguchi 2009; Chavez-Dozal et al. 2012). Metabolic activity was measured by the XTT reduction assay (Pierce et al. 2010). In brief, 0.010 mol/L menadione (Sigma-Aldrich, St. Louis, MO) stock solution (diluted in acetone) was mixed with XTT/Ringer's lactate solution (0.5 g of XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide} from Sigma diluted in 1 L of 1× PBS or Ringer's lactate solution) at a final concentration of 1 μmol/L. An aliquot of the XTT/Ringer's/menadione solution was then added to each prewashed well. The plates were covered in aluminum foil and incubated for 2 h at 28 °C. If the XTT is effectively reduced by metabolically active cells, the original clear solution is transformed into an orange solution that can be measured at A_{490}. For CV and XTT assays, experiments were performed three times independently (biological replicates), each in quadruplicate (technical replicates) including inoculated sterile LBS as a negative control.

For dry cell mass determination, biofilms were formed in 96-well microplates and planktonic bacteria were removed after 24 h of incubation (as described previously; Taff et al. 2012). Biofilms were dried for 30 min at room temperature and then were disrupted by scraping with a sterile spatula and diluted into 500 μL of sterile water. The biofilm suspension was filtered through a preweighted filter (0.45 μm) and dried in an incubator at 105 °C for 2 h, after which the filter was weighed again. The dry mass of the biofilm was calculated based on mass differences between the control and samples.

For enumeration of bacteria in biofilms, the biofilm assay plates were inoculated, incubated, and washed as described for the CV and XTT assays. Each individual well was cut with scissors and 100 μL of 1× PBS was added. The well (plus the PBS) was placed into a separate 10-mL tube containing 1.9 mL of 1× PBS. The sample was sonicated for 5 s at 30 % power (higher sonication times compromised cell viability of some strains). The sample was plated in triplicate onto LBS plates and incubated for 24 h at 28 °C. Viable counts of colony-forming units were performed. For each strain, the experiment was performed in triplicate.

We additionally performed a semiquantitative method to measure biofilm formation by observation of wrinkled colony

development as described previously (Merrit et al. 2005) with minor modifications. In brief, an aliquot of overnight cultures was subcultured in 5 mL of fresh LBS at a 1:100 dilution and grown to an A_{600} of 0.2. After incubation, 1 mL of culture was pelleted and washed twice with 1× PBS, and resuspended in 1 mL of 1× PBS. Ten-microliter subsamples were spotted onto a fresh LBS plate (three spots per plate) and incubated for 24 h at 28 °C. Morphology and spot size were observed, and light micrographs of colonies were acquired using an inverted microscope (Micromaster digital inverted microscope with infinity optics, Fisher Scientific, Waltham, MA). The diameter of the colony was recorded digitally using the data acquisition software Micron 2.0.0 (Westover Scientific, Milpitas, CA). This experiment was performed in triplicate for each strain.

Results were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc or Tukey comparison post-test. Differences between groups were considered to be significant at a P value of <0.05. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

Findings

In vitro studies of biofilms have been increasing in number over the last decade. *Vibrio* biofilms play an important role in the environment and have been studied in the laboratory for over a decade (Yildiz and Visick 2008). There are multiple assays that have been proposed for quantification of *Vibrio* biofilms; for example, crystal violet is one of the most commonly used methods (Ray et al. 2011; O'Toole 2011; Kuhn et al. 2003; Chowdhury et al. 2011), and consists of a colorimetric assay where crystal violet solution (water or ethanol based) is used to stain cells and their extracellular matrices. The amount of CV absorbed by the biofilm is quantified by optical density readings of dissolved crystal violet, which is directly proportional to the biofilm mass. An alternative method consists of weighing the dried biofilm. This is one of the techniques used to calculate the total amount of biofilm but does not account for cell viability within the biofilm. The colony-forming unit determination assay (CFU counts) is a labor-intensive method that is solely based on cell viability. Moreover, the recently proposed method of observation of wrinkled colonies provides a more reliable method of quantifying biofilm development, which also allows the evaluation of the tri-dimensional structure and patterning of a particular *Vibrio* biofilm former. Some limitations of CFU and wrinkled colony development assays include lengthy assay time and requirements for previous adjustments to ensure reproducibility. In addition, strains with growth defects are usually difficult to analyze and cells in the viable but non-culturable state will not be detected (McDougald et al. 1998). For those types of assays that require removal of adherent biofilms (dry cell measurement and CFU counting), removal of cells may be inconsistent between samples.

An alternative method that has been widely used and has been proven to be especially useful for the study of fungal biofilms (in particular *Candida albicans*) is a colorimetric assay based on cellular viability involving the use of tetrazolium salts (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide, or XTT) and measurement of its orange-colored formazan product (due to activity of succinoxidase and cytochrome P450 enzymes). Since this assay is easy to perform, we included this analysis in quantification of *Vibrio* biofilms to combine measurements of cell viability with biofilm mass.

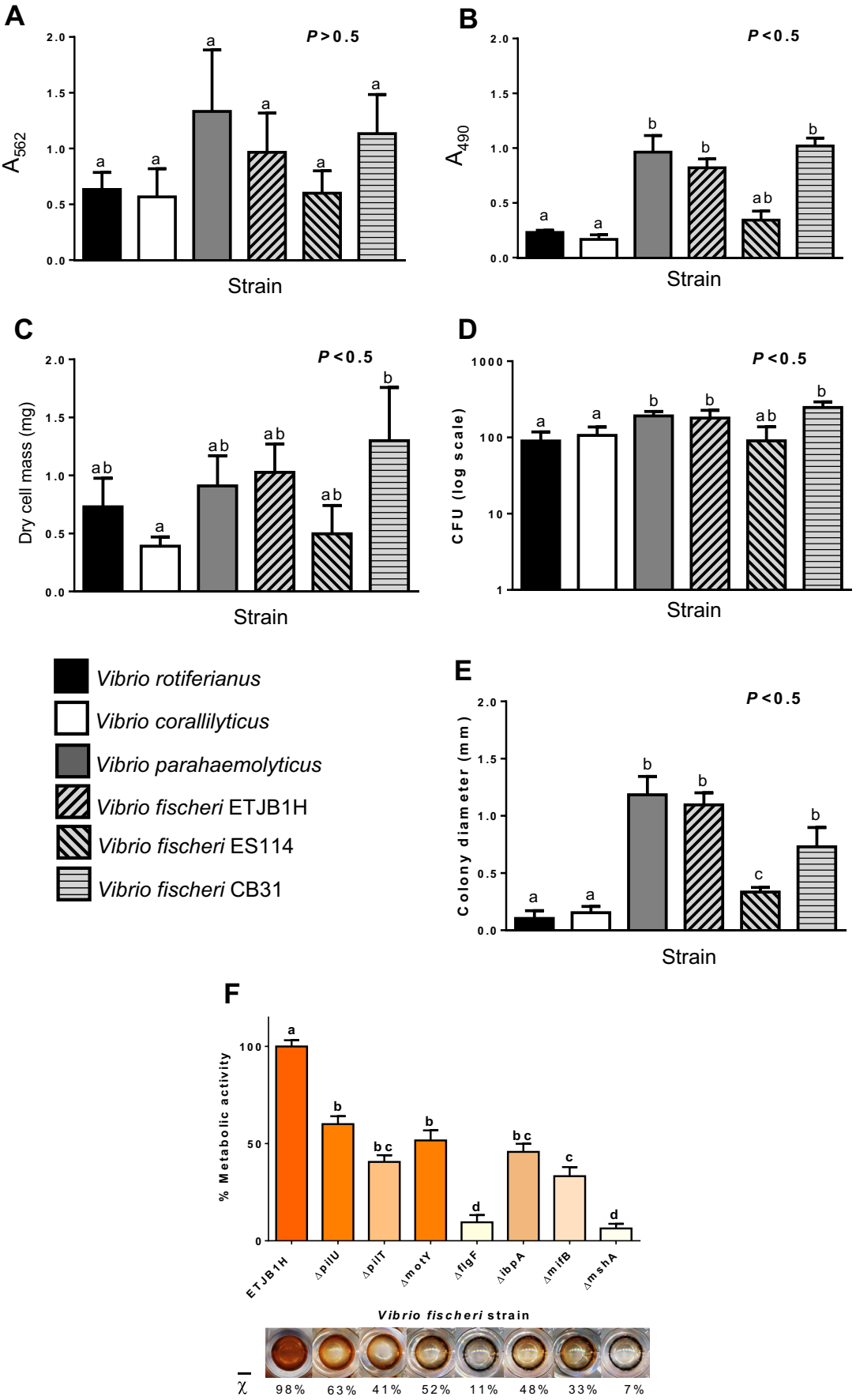
Since it is not advisable to conduct biofilm formation experiments on strains with growth defects, we performed growth curves on all the strains used in this study and none of them exhibited defects in growth. Additionally, for those biofilm formation experiments done in 96-well plates (including crystal violet and XTT), we measured the optical density (A_{600}) of the plate after incubation and prior to addition of either CV or menadione/Ringer's. All strains were between an A_{600} range of 5–6.

Among the assays tested, the most time consuming (but accurate and reproducible) were the CFU counting and the wrinkled colony development, whereas the crystal violet and dry cell mass assays were the most inaccurate and least reproducible (Table 1). Additionally, the XTT assay was the most reliable, the least time consuming, and the least costly. Figure 1 shows a more detailed comparison of the assays tested and their variability according to statistical differences (P values). An additional advantage of the use of metabolism-based assays (XTT assay) was that it allowed comparison of biofilm formation efficacy of mutant cells with the parental strain. This is illustrated in Fig. 1f, where different *V. fischeri* mutants in genes that have been reported to be important for

Table 1 Summary of the different methods used to quantify biofilms

Assay	Accuracy	Reproducibility	Time
XTT assay	++++	++++	+
Crystal violet	+	+	+
Dry cell mass	++	++	++
CFU count	+++	+++	++++
Wrinkled colony development	++++	++++	+++

Accuracy represents whether the data was consistent among technical replicates, as well as whether differences between the strains were significant ($P < 0.05$). Reproducibility was determined by the coefficient of variation (CV) for each set of data between biological replicates; ++++ = CV <0.1, +++ = CV of 0.1–0.15, ++ = CV of 0.15–0.2, and + = CV of >0.2. Time accounts for both the total length of the protocols and the amount of labor required for each (++++ representing the most time-consuming protocols)



◀ **Fig. 1** Comparison of five in vitro biofilm quantification methods for *Vibrionaceae* biofilms. Each graph represents the data of the average (with *error bars* indicating standard deviations) of three independent experiments (biological replicates). *Different letters* on the *abscissa* denote significant differences between groups according to the Tukey post hoc comparison. *P* values indicate significant ($P < 0.5$) or non-significant ($P > 0.5$) overall differences according to the one-way ANOVA test. Absorbancies (A_{562} and A_{490}) of biofilms using **a** crystal violet (CV) or **b** XTT assay, respectively. **c** Biofilm quantification via dry cell mass measurement. **d** Colony-forming unit (CFU) determination of cells in biofilms formed in 96-well microplates. **e** Diameter of wrinkled colonies measured after 24 h of incubation. **f** Metabolic activity of wild-type (ETJB1H) and mutant *Vibrio fischeri* strains. Δ*pilT*, Δ*pilU*, and Δ*mshA* are type IV pilus mutants; Δ*flgF* and Δ*motY* are mutants in flagellum assembly and functionality; Δ*ibpA* is a mutant of a chaperonin responsible for the heat stress response, and Δ*mifB* is a mutant of the magnesium-dependent induction response. Metabolic activity is calculated as percentage in relation to *A* readings (A_{490}) of the wild-type parental strain. *Different letters* indicate significant differences according to the Tukey post hoc comparison test. *Wells* indicate the representative image of the intensity of the orange product as a result of formazan production by each biofilm. $\overline{\chi}$ represents the median value of the metabolic activity (in percentage)

biofilm development (but that are not defective in planktonic growth) were compared based on metabolic activity.

For the *Vibrionaceae* strains tested, we found that the XTT assay is the most reproducible and efficient method for measurement of biofilm biomass. The observation of development of wrinkled colonies could be used as a complementary test as it allows observation of the tri-dimensional structure of the biofilm and complements the colorimetric approach.

Acknowledgments The authors would like to thank Dr. Maurizio Labbate for providing the *Vibrio rotiferianus* strain. A.A.C.D. was supported by the NMSU National Institutes of Health RISE to the doctorate (NIH NIGMS R25GM061222), Institutional Research and Career Development Award, and the Academic Science Education and Research Training Program (IRACDA-ASERT, NIH-K12GM088021).

References

Ariyakumar DS, Nishiguchi MK (2009) Characterization of two host specific genes, mannose sensitive hemagglutinin (mshA) and uridyl phosphate dehydrogenase (UDPH) that are involved in the *Vibrio fischeri-Euprymna tasmanica* mutualism. FEMS Microbiol Lett 299:65–73

Chavez-Dozal AA, Hogan D, Gorman C, Quintanal-Villalonga A, Nishiguchi MK (2012) Multiple *Vibrio fischeri* genes are involved in biofilm formation and host colonization. FEMS Microbiol Ecol 81:562–573

Chowdhury PR, Boucher Y, Hassan KA, Paulsen IT, Stokes HW, Labbate M (2011) Genome sequence of *Vibrio rotiferianus* strain DAT722. J Bacteriol 193(13):3381–3382

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Kuhn DM, Balkis M, Chandra J, Mukherjee PK, Ghannoum MA (2003) Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. J Clin Microbiol 41(1):506–508

McDougald D, Rice SA, Weichart D, Kjelleberg S (1998) Nonculturability: adaptation or debilitation? FEMS Microb Ecol 25(1):1–9

Merrit JH, Kadouri DE, O'Toole GA (2005) Growing and analyzing static biofilms. Curr Protocol Microbiol Chapter 1:Unit 1B.1

O'Toole GA (2011) Microtiter dish biofilm formation assay. J Vis Exp 30(47). doi: 10.3791/2437.

Pierce CG, Uppuluri P, Tummala S, Lopez-Ribot JL (2010) A 96 well microtiter plate-based method for monitoring formation and antifungal susceptibility testing of *Candida albicans* biofilms. J Vis Exp 44: 2287

Ray VA, Morris AR, Visick KL (2011) A semi-quantitative approach to assess biofilm formation using wrinkled colony development. J Vis Exp 7(64):e4035. doi:10.3791/4035

Taff HT, Nett JE, Andes DR (2012) Comparative analysis of *Candida* biofilm quantitation assays. Med Mycol 50:214–218

Watnick P, Kotler R (2000) Biofilm, city of microbes. J Bacteriol 182: 2675–2679

Yildiz FH, Visick KL (2008) *Vibrio* biofilms: so much the same yet so different. Trends Microbiol 17:109–118

Chapter 7

Impact of Metabolomics in Symbiosis Research

Alba Chavez-Dozal and Michele K. Nishiguchi

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66631

Abstract

In symbiotic associations, there is a constant molecular complexity that allows establishment and maintenance of the relationship. Metabolomic profiles have enabled researchers to explain symbiotic associations in terms of their underlying molecules and interactions between the symbiotic partners. In this review, we have selected studies on symbioses as examples that have helped to explain the metabolic integration of bacterial symbionts and their hosts in an effort to understand the molecular fingerprint of animal-microbial symbioses.

Keywords: symbiosis, mutualism, metabolomics, co-clustering analysis

1. Introduction

The intimate association between two organisms is a very complex biological phenomenon; nevertheless, it is a very common way of life for every living organism on Earth. Symbiotic associations with one or many phylogenetically different organisms provide a fascinating view into how symbionts adapt and co-evolve. As Chaston and Douglas beautifully described in their comprehensive review [1], the omics revolution has transformed our ability to understand symbiotic associations at the molecular level. Researchers have adopted multiple techniques with great fervor in an effort to decipher the basis and complexity of symbiotic associations. Until recent years, the molecular pathways of symbiotic associations could only be studied in the context of genetic changes (transcriptomic studies) and protein profiles (proteomics); however, it is very likely that the establishment of a mutualistic association involved multiple evolutionary changes in the biochemistry and metabolic network of all the partners involved in the symbiosis [1]. Omics biology brings challenges and opportunities; one of the recent advances is the ability to construct a molecular metabolic catalog of an organism within a symbiotic association.

Metabolomics refers to the analytical approach used to study different cell products ("chemical fingerprints") that help to understand the physiological state of an organism [2].

In this section, we provide a comprehensive description of four experiments where the approach of metabolomics was selected in a particular type of animal-microbial symbiosis, in order to answer specific questions in symbiosis research.

2. Exemplars of metabolomic approaches in symbiosis research

2.1. Inferring metabolic interactions in arbuscular mycorrhizal symbiosis

Our exemplar of metabolomics studies of microbe-plant interactions is a set of observations by Schweiger et al. [3] that describe species-specific leaf metabolic responses to arbuscular mycorrhiza (AM) [4]. Arbuscular mycorrhiza is a unique symbiotic association between root arbuscular mycorrhizal fungi (AMF) and plants [4]. This is an ancient and widespread association where the fungus improves water uptake to the host plant, and in return the fungus receives plant carbohydrates. The fungus is restricted to the roots of the plant; however, the biochemical pathways and the involvement of exchanged substances are reflected on systemic root tissues affecting the chemical composition of plant tissues (defined as "phytometabolome") [4].

Comparative studies conducted on five different plant-AMF associations demonstrate that foliar metabolome is highly plant-species-specific, with low degrees of conservation across species. The experimental design was crucial to the success of this analysis, with the metabolome analysis performed on leaves of five plant species exposed to the worldwide distributed AMF *Rhizophagus irregularis*. Furthermore, the study took into account the implications of metabolite fluctuation at different leaf developmental stages and plant-reproductive status. Additionally, mycorrhizal plants were compared with control plants that received a sterilized inoculum. The results from this study indicate the high specificity of plant metabolome responses to the same AMF colonization; among the most striking findings indicate that metabolomics responses related to phosphate uptake, citric acid cycle, and amino acids were species-specific [3]. **Figure 1** summarizes the most important findings of this interesting study.

2.2. Metabolomic profile of the ryegrass-endophyte symbiosis

Along the lines of microbe-plant interactions, there is an interesting study conducted by Cao et al. [5] that is of particular relevance for symbiosis research. The metabolomics profile of perennial ryegrass (*Lolium perenne*) infected with endophytic fungus (*Neotyphodium lolii*) provided understanding of regulatory biochemical mechanisms for the production of beneficial alkaloids.

N. lolii is a naturally occurring fungus whose complete cycle occurs within perennial ryegrass. The fungus grows between the cells of the host plant drawing nutrients from it, and in return, the endophyte produces chemical compounds that provide resistance to drought, pests, and protection from overgrazing. Therefore, the aim of this study was to gather metabolomics information and combine it with microarray data in order to obtain a better understanding of

http://dx.doi.org/10.5772/66631

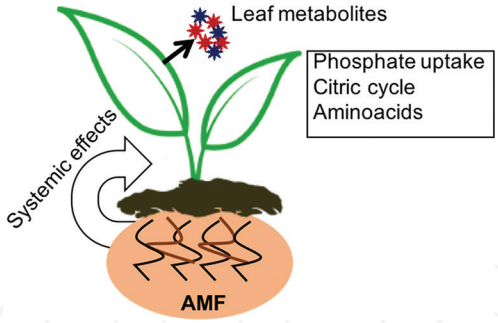


Figure 1. Summary of findings on the leaf phytometabolome when plants are exposed to the same arbuscular mycorrhizal fungi (AMF). Leaf metabolites detected included carbohydrates, organic acids, amino acids and derivatives, cyclic polyols, and sugar alcohols. Metabolites are differentially regulated primordially affecting the phosphate and citric acid cycles.

the biochemical mechanisms involved in the cross talk between partners, with the eventual purpose of achieving genetic manipulation of beneficial metabolite production (in particular manipulation of alkaloids).

Twenty-four perennial ryegrass samples comprising three tissue types (immature leaves, blades, and mature leaves) were examined of both endophyte-infected plants and endophyte free as a control. Targeted metabolomics analysis was used as the quantitative approach that provided identities of 70 metabolites based on the available databases of reference compounds. The use of targeted metabolomics in combination with microarray data provided better identification and classification Accuracy of compounds, as well as greater insights into the dynamics and fluxes of the newly identified metabolites. Results of this comprehensive study included the identification of accumulated alkaloids in the mature tissues of endophyte-infected ryegrass, and the co-clustering analysis of microarray data-identified genes with distinctive expression patterns which coincide with the pattern of alkaloid accumulation [5]. **Figure 2** summarizes the findings of this study. Results of this study indicate that co-clustering analysis is not a straightforward task no matter what kind of algorithm is used, and that the integration of transcriptomics and metabolomics can generate noisy data. However, this study demonstrated that co-cluster analysis could be a comprehensive choice to gain a more complete understanding of a complex biological system involving two entirely different taxa that are intertwined in their metabolic capabilities.

2.3. Metabolomic profile of symbiotic protection against pathogens

It is believed that specific strains from the gut microbiota can influence host immunity and protect from infection by pathogenic bacteria. One example is the early and prevalent gut colonizer *Bifidobacterium*, which is considered part of the healthy normal gut flora. It is believed that different strains of *Bifidobacterium* protect against enteropathogenic *Escherichia coli* O157:H7 infection in mice; however, the potential molecular and cellular mechanisms underpinning this protective effect are still under investigation [6].

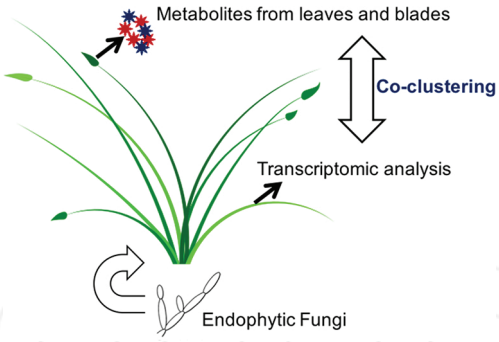


Figure 2. Summary of the study conducted by Cao et al. Co-clustering analysis of microarray and metabolomics data on endophytic-infected ryegrass indicate a set of genes and metabolites that are important for alkaloid production.

One study conducted by Fukuda et al. [7] used a combined "omics" strategy in an effort to gain a better understanding of the protective effect of *Bifidobacterium* over its mice host. Experiments designed comprised mice infected with different species of the symbiotic bacterium *Bifidobacterium* (including *B. longum* and *B. adolescentis*) and the pathogen *E. coli* O157:H7. The life span of co-infected mice was observed and transcriptomic and metabolomic profiles were conducted. **Figure 3** diagrams the experimental design of this study. This sophisticated analysis included a combination of sequencing, the platform used for metabolite detection was HPLC-MS (high-performance liquid chromatography-mass spectrometry) and for the analysis of products, the dataset was subjected to a multivariate analysis method named PLS (partial least squares) projection to latent structures. Typical data-processing flow included detection of signal peaks and normalization of dataset to generate a matrix of the products detected. For their statistical analysis, the method selected was PCA (principal component analysis) and CL (cluster analysis).

Results from this study indicate that mice bearing the strain *B. longum* survived, whereas those infected with *B. adolescentis* died. Metabolomic profiles between the two treatments revealed that the concentration of fatty acids (acetic acid in particular) was significantly elevated in those mice that survived *E. coli* infection. Furthermore, mice that survived showed an increased expression of genes involved in ATP-binding-cassette carbohydrate transporters [7]. Observations from the study suggest that the elevated production of acetic acid improved intestinal defense, thereby enhancing the barrier function of colon epithelial cells inhibiting the transport of *E. coli* toxins.

2.4. Metabolomics of a beneficial marine bacterium

The marine luminescent bacterium *Vibrio fischeri* establishes a symbiotic association with numerous sepiolid squids and monocentric fishes. *V. fischeri* infects a specialized light organ in the mantle (body) cavity of host squids and produces bioluminescence that is used by its host to avoid predation in a behavior known as counterillumination. In return, the squid host provides an enriched habitat for *Vibrio* to reproduce and to form bacterial communities of

monospecies biofilms. The ability of *V. fischeri* to form a biofilm in the light organ of its squid host plays a central role in establishment and maintenance of the symbiotic association. This interesting symbiotic association has been the center of attention of many researchers, and has been investigated for more than 25 years; however, as indicated for other examples of mutualistic associations, the molecular basis of the squid-*Vibrio* symbiosis is still obscure.

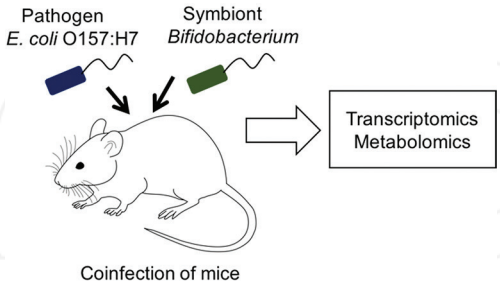


Figure 3. Summary of the experiment conducted by Fukoda et al. Mice were coinfected with beneficial strains of *Bifidobacterium* and the pathogenic strain of *Escherichia coli* O157:H7. Combined transcriptomic and metabolomic profiles revealed an increase in acetate and fructose transporters in those mice that survived lethal infection.

In a recent study conducted by Chavez-Dozal et al. [8], both proteomic and metabolomic profiles were performed in parallel in strains of *V. fischeri* in their biofilm form and compared to profiles of free-living (or planktonic) *V. fischeri* cells of the same strain. The main objective of this study was to obtain a comprehensive profile of the molecular components to provide the first meta-proteome profile of biofilms that are important for establishment of this mutualistic association. A summary of this study is illustrated in **Figure 4**.

Biofilms are a complex microbial community composed of cells encased within a self-produced exopolymeric matrix. Expression profiles of biofilm communities reveal the composition of the matrix, which include a combination of lipids, polysaccharides, proteins, and DNA [9, 10].

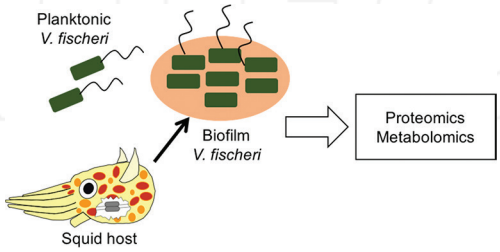


Figure 4. Summary of the experiment conducted by Chavez-Dozal et al. [5]. Proteomic and metabolomic profiles were performed in planktonic cells and biofilm communities of the same strain of *Vibrio fischeri*. Results revealed an upregulation of biofilm matrix components and molecules related to multiple stress responses.

Results of this study revealed a time-resolved picture of approximately 100 proteins and 200 metabolites present in the biofilm state of *V. fischeri*. The most important components found in this study include proteins, sugars, and molecules that form part of the exopolysaccharide matrix of biofilms; surprisingly, an increased concentration of intermediates of the glycolysis pathway was found to be prevalent during the biofilm state [8]. Results from this study suggest that molecules involved in the construction of the biofilm matrix are essential to bacterial community formation, a process that has been known to activate stress responses such as upregulation of alternative anaerobic pathways. The reported findings of this study have broad implications for *V. fischeri* ecology, since many of the symbiosis-regulated genes are not yet described. The combination of proteomics and metabolomics has therefore provided a link between protein regulation and function during different phases of the symbiosis, improving our understanding of the mechanisms that are important for successful host colonization.

3. Concluding remarks

Metabolomic approaches are increasingly selected for multiple purposes of symbiosis research. Although other "omic" approaches are needed to understand molecular function in symbiotic associations, the emerging use of metabolomics provides a new level of biochemical sophistication. The different examples provided in this mini review are only some of the pillar studies that included the use of either metabolomics or a combinational analysis of metabolomics with transcriptomics/proteomics of different mutualistic systems; however, many more studies are in progress using metabolomics profiles to define and characterize molecular and biochemical pathways that are important for establishment and persistence of symbiotic associations. The advancement of technologies that allows higher resolution of minute concentrations of proteins and their modulation will expand the area of metabolomics research and will enable a better perspective of the physiological state of organisms as single entities (otherwise known as the holobiome).

Author details

Alba Chavez-Dozal and Michele K. Nishiguchi*

*Address all correspondence to: nish@nmsu.edu

Department of Biology, New Mexico State University, Las Cruces, NM, USA

References

[1] Chaston J, Douglas AE. Making the most of "omics" for symbiosis research. Biol Bull; 2012;**223**:21–9. doi: 10.1111/j.1365-2133.2012.10859.x.

[2] Tyagi S, Raghvendra, Singh U, Kalra T, Munjal K. Applications of metabolomics, A systematic study of the unique chemical fingerprints: an overview. Int J Pharma Scie Rev and Res; 2010;**3**(1):019.

[3] Schweiger R, Baier MC, Persicke M, Muller C. High specificity in plant leaf metabolic responses to arbuscular mycorrhiza. Nat Comm; 20114;**5**:3886. doi: 10.1094/MPMI-05-14-0126-R.

[4] Schweiger R, Muller C. Leaf metabolome in arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol; 2015;**26**:120–6. doi: 10.1016/j.pbi.2015.06.009.

[5] Cao M, Johnson L, Johnson R, Koulman A, Lane GA, Rasmussen S. Joint analyses of transcriptomic and Metabolomic data to probe ryegrass-endophyte symbiosis. In: Popay A, Thom E (eds). Proceedings of the 6th international symposium on fungal endophytes of grasses. Christchurch, New Zealand, December 2007.

[6] Sugahara H, Odamaki T, Fukuda S, Kato T, Xiao J, Abe F, Kikuchi J, Ohno H. Probiotic *Bifidobacterium longum* alters gut luminal metabolism through modification of the gut microbial community. Sci Rep; 2015;**5**:13548. doi:10.1038/srep13548.

[7] Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL, Suzuki T, Taylor TD, Itoh K, Kikuchi J, Morita H, Hattori M, Ohno H. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature; 2011;**469**:543–7. doi: 10.1038/nature09646.

[8] Chavez-Dozal A, Gorman C, Nishiguchi MK. Proteomic and metabolomics profiles demonstrate variation among free-living and symbiotic *Vibrio fischeri* biofilms. BMC Micro; 215;**15**:226. doi: 10.1186/s12866-015-0560-z.

[9] Hobley L, Harkins C, MacPhee CE, Stanley-Wall NR. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. FEMS Microbiol Rev; 2015;**39**(5):649–69. doi: 10.1093/femsre/fuv015.

[10] Jiao J, D'haeseleer P, Dill BD, Shah M, VerBerkmoes NC, Hettich RL, Banfield JF, Thelen MP. Identification of biofilm matrix-associated proteins from an acid mine drainage microbial community. Appl Env Microbiol; 2011;**77**(15):5230–7. doi: 10.1128/AEM.03005-10.

Integrative and Comparative Biology

Integrative and Comparative Biology, volume 57, number 1, pp. 7–17
doi:10.1093/icb/icx004

Society for Integrative and Comparative Biology

INVITED PAPER

Broadening Participation in the Society for Integrative and Comparative Biology

Cheryl A.D. Wilga,[1,*] Michele K. Nishiguchi[†] and Brian Tsukimura[‡]

*Biological Sciences Department, University of Alaska Anchorage, 3101 Science Circle, Anchorage, AK 99508, USA; †Department of Biology, New Mexico State University, PO Box 30001, MSC 3AF, Las Cruces, NM 88003, USA; ‡Department of Biology, California State University, Fresno, 2555 E. San Ramon Avenue, Fresno, CA 93740, USA

[1]E-mail: cwilga@alaska.edu

Synopsis The goal of the Society for Integrative and Comparative Biology's Broadening Participation Committee (SICB BPC) is to increase the number of underrepresented group (URG) members within the society and to expand their capabilities as future researchers and leaders within SICB. Our short-term 5-year goal was to increase the recruitment and retention of URG members in the society by 10%. Our long-term 25-year goal is to increase the membership of URG in the society through recruitment and retention until the membership demographic mirrors that of the US Census. Our plans to accomplish this included establishment of a formal standing committee, establishment of a moderate budget to support BPC activities, hosting professional development workshops, hosting diversity and mentor socials, and obtaining grant funds to supplement our budget. This paper documents broadening participation activities in the society, discusses the effectiveness of these activities, and evaluates BPC goals after 5 years of targeted funded activities. Over the past 5 years, the number of URG members rose by 5.2% to a total of 16.2%, members who report ethnicity and gender increased by 25.2% and 18%, respectively, and the number of members attending BPC activities has increased to 33% by 2016. SICB has made significant advances in broadening participation, not only through increased expenditures, but also with a commitment by its members and leadership to increase diversity. Most members realize that increasing diversity will both improve the Society's ability to develop different approaches to tackling problems within integrative biology, and help solve larger global issues that are evident throughout science and technology fields. In addition, having URG members as part of the executive committee would provide other URG members role models within the society, as well as have a voice in the leadership that represents diversity and inclusion for all scientists.

Introduction

The Society for Integrative and Comparative Biology (SICB) is one of the largest and most prestigious professional associations of its kind. The Society is dedicated to promoting the pursuit and public dissemination of information relating to biological sciences. SICB takes pride in the fact that one of the Society's focal points is to support student members, and that the organization is fundamentally committed to the advancement and development of early career investigators through its programs, meetings, and journal publications (Integrative and Comparative Biology).

The main goal of SICB's Broadening Participation Committee (BPC) is to increase the number of underrepresented group (URG) members within SICB and to expand their capabilities as future researchers within the SICB divisions. SICB has a long-standing mission and commitment to increasing the diversity of URG members, and recognizes the importance of engaging this group of scientists to address future needs within various biological disciplines. Integration among a number of different and diverse disciplines is crucial for our understanding of complex biological questions, and the capability of enabling our future scientists to tackle innovative and interesting ideas regarding organismal variation is in part driven by approaching questions from unique backgrounds and experiences (Schmidt 2010; Kendall 2011). SICB's Broadening Participation activities provide that platform to nurture students,

Advanced Access publication July 5, 2017

postdoctoral researchers, and beginning young investigators, which will create a community that will enhance their participation in the society and beyond (Ely and Thomas 2001). We hope that our activities will equip these young scientists with the toolkit needed to be successful within their careers and eventually become leaders in their fields, utilizing their background and experiences to become our future innovators and teachers (Goode 2004).

The BPC proposed a 5-year Strategic Plan in 2010 to broaden participation within the Society. The proposed objectives were aimed at coalescing and enhancing the experience of participants with new activities that complement those already in place. We established a program that will develop and increase participation of URG (URGs = including minorities, those with disabilities, first generation college attendees, and veterans) by publicizing the benefits of being an active SICB member and encouraging them to participate in leadership roles (Gardner 2013). Our short-term 5-year goal was to increase the recruitment and retention of URG members in the society by 10%. Our long-term 25-year goal is to increase the membership of URG in the society through recruitment and retention until the membership demographic mirrors that of the US Census. Our plans to accomplish this included establishment of a formal standing committee, establishment of a budget to support BPC activities, offering a variety of professional development workshops, hosting diversity socials and obtaining grant funds to supplement our budget. This paper documents broadening participation activities in SICB, discusses the effectiveness of these activities and assesses BPC goals after 5 years of targeted activities.

The importance of diversity in science

There is a deficit of URG members in scientific societies, which is likely due to the deficit of URGs graduating from universities (Smith 1991; Xu and Martin 2011). Diverse learning environments are most effective with all members benefiting from the increased awareness and broader perspectives of its members (Gurin et al. 2004; Peckham et al. 2007; Holly 2013). Thus, groups involving diverse members provide greater critical analyses of problems and solve them in more innovative ways (McLeod et al. 1996). If the trend in the United States showing an increased URG population continues, the majority of children born in the 21st century will belong to URGs, which are underrepresented in STEM fields (NSF 2014). To be responsive to the rapidly changing demographics of the United States, there is a critical need to broaden participation of URGs in STEM fields, such as those represented in SICB. To be effective in promoting the pursuit and dissemination of relevant and timely biological information to the public, membership demographics in SICB must reflect that of society.

Early history of broadening participation in SICB

There are several SICB committees that provide opportunities for students at annual meetings prior to 2002 through the present. The Student/Postdoctoral Affairs Committee hosts a Student First Timer workshop explaining how to get the most out of your SICB meeting, and a topical workshop at each annual meeting. The Student Support Committee is charged with overseeing activities related to student support, including the Charlotte Mangum awards that cover housing or registration, as well as individual research awards (Grants-In-Aid-of-Research). The Education Council arranges for undergraduate students to display their posters near the plenary session at annual meetings. However, until 2002 there were no efforts at increasing membership of URGs. Then-President Marvalee Wake created the BPC in 2002, with the goal of increasing diversity in SICB and fields of Integrative and Comparative Biology. From 2005 until 2009, local faculty and their undergraduate students, along with high school students and their teachers, were recruited to attend and present posters at the annual meeting. Registration and lodging were provided for up to 10 individuals each year. SICB graduate student members were then recruited to mentor the local undergraduate and high school students throughout the meeting. A complimentary breakfast was held on the first day of the meeting for self-identified ethnic minorities to network, and where National Science Foundation program directors spoke about funding opportunities. These were good attempts to introduce science in the SICB to local URG undergraduate and high school students and faculty, but it was not clear how many of these local recruits remained members and attended future annual meetings. The Then-BPC Chair Patricia Hernandez initiated a mechanism for self-identification of ethnicity and gender on annual meeting registration forms for the 2009 annual meeting and the 2010 annual membership renewal. These data continue to be collected on annual (online) membership forms as well as for annual conference attendees.

Formalizing the BPC in SICB

The Executive Committee recognized the need for greater diversity and in 2009 formally recognized

the BPC as a standing committee of the SICB where the Chair was elevated to the SICB Executive Committee, which permits participation in voting on SICB activities, including in budget discussions. This enabled the BPC, which included the authors (Cheryl Wilga, Then-BPC Chair, Brian Tsukimura, Then-Program Officer and Then-BPC member, and Nish Nishiguchi, Then-BPC Member), to develop a regular budget ($10,000 in 2010 that was gradually increased to $15,000 in 2012). SICB students indicated a need for financial support to attend the annual meeting; therefore, the BPC established travel awards to allow more URG members to attend the annual meeting. The BPC started offering two workshops geared toward the needs of URG to provide professional development opportunities. One of these workshops focused on junior members (graduate students) and the other targeted senior members (postdocs and faculty). The BPC supports a meet and greet social on the first day of the annual meeting to allow travel award fellows to meet their cohorts, past cohorts, mentors and the BPC members. The BPC also supports a Diversity Social near the end of the annual meeting where travel award checks are distributed and where travel award fellows can meet and network with other SICB members, including past and current executive committee members, program directors from the National Science Foundation and other invited guests. These activities were initiated at the 2011 annual meeting and continued through the 2017 meeting, and were very successful.

SICB resources

The SICB leadership is very committed to broadening participation in the society and has many resources already in place that enable BPC activities to be implemented effectively. The BPC has retained its annual budget of $15,000. The SICB webmaster, Mr Birenheide, designs and maintains the webpage and online resources for announcements and travel award applications. The SICB has a very effective administrative team (Burke Associates Inc.) that organizes the annual meetings. They are instrumental in allocating rooms for the workshops and socials (along with the Program Officer), organizes food orders for the socials, manage the budget and print award checks. The elevation of BPC to the SICB Executive Committee ensures that all of these activities will be retained in the future. These resources are at the disposal of the BPC who are a dedicated group of volunteer members that have a vision for broadening participation within the Society. The BPC recognizes the importance of creating and sustaining a diverse community of scientists, and is willing to commit time and energy to ensure that our goals are not only embraced but also achieved.

SICB BPC objectives

The main goal of the BPC is to create a culture where members from URGs have access to a number of resources, such as mentors, workshops, funding for travel to annual meetings and a sense of community within the society. By building upon SICB's base of URG members, immediate feedback can be obtained on the needs of those members who are currently at different stages of their careers (graduate students, postdoctoral fellows, junior faculty and senior faculty). The BPC can assess what resources fit the needs for each level of our URG member pool, and reinforce SICB's ability to ensure their success. The BPC census of SICB composition has indicated that the URG numbers show a dramatic decline at the level of postdoctoral and assistant professors (Fig. 1); yet causes for this decline are not apparent from these data. Our objectives include tracking these data to attempt to identify and address as many of the causes as possible, and establish long-term solutions that will enable postdoctoral and assistant professor members to persist and grow within the SICB.

Eight BEST principles were identified as most successful in recruiting and retaining URG in other professional societies and in STEM fields (Wilson and Haynes 2002; Pandya et al. 2007; Payton et al. 2012). The goals of the BPC align closely with the BEST principles: 1) institutional leadership, 2) targeted recruitment, 3) engaged faculty, 4) personal attention, 5) peer support, 6) enriched research experience, 7) bridging to the next level and 8) continuous evaluation. Pandya et al. (2007) identified one pervasive need—financial support—that can easily be addressed by scientific societies. The BPC goals align well with these design principles and have implemented activities that address some of these, which include: 1) provide support for attending our annual meeting in the form of travel awards, which will promote and sustain a URG cohort that in turn will be our future leaders in the society and beyond; 2) increase awareness of broadening participation and building community by promoting events at the annual meeting (socials and workshops); 3) offer workshops that address issues specific for URG members (i.e., career development, leadership, teaching and outreach); 4) recruit new URG members and promote the society to other societies in

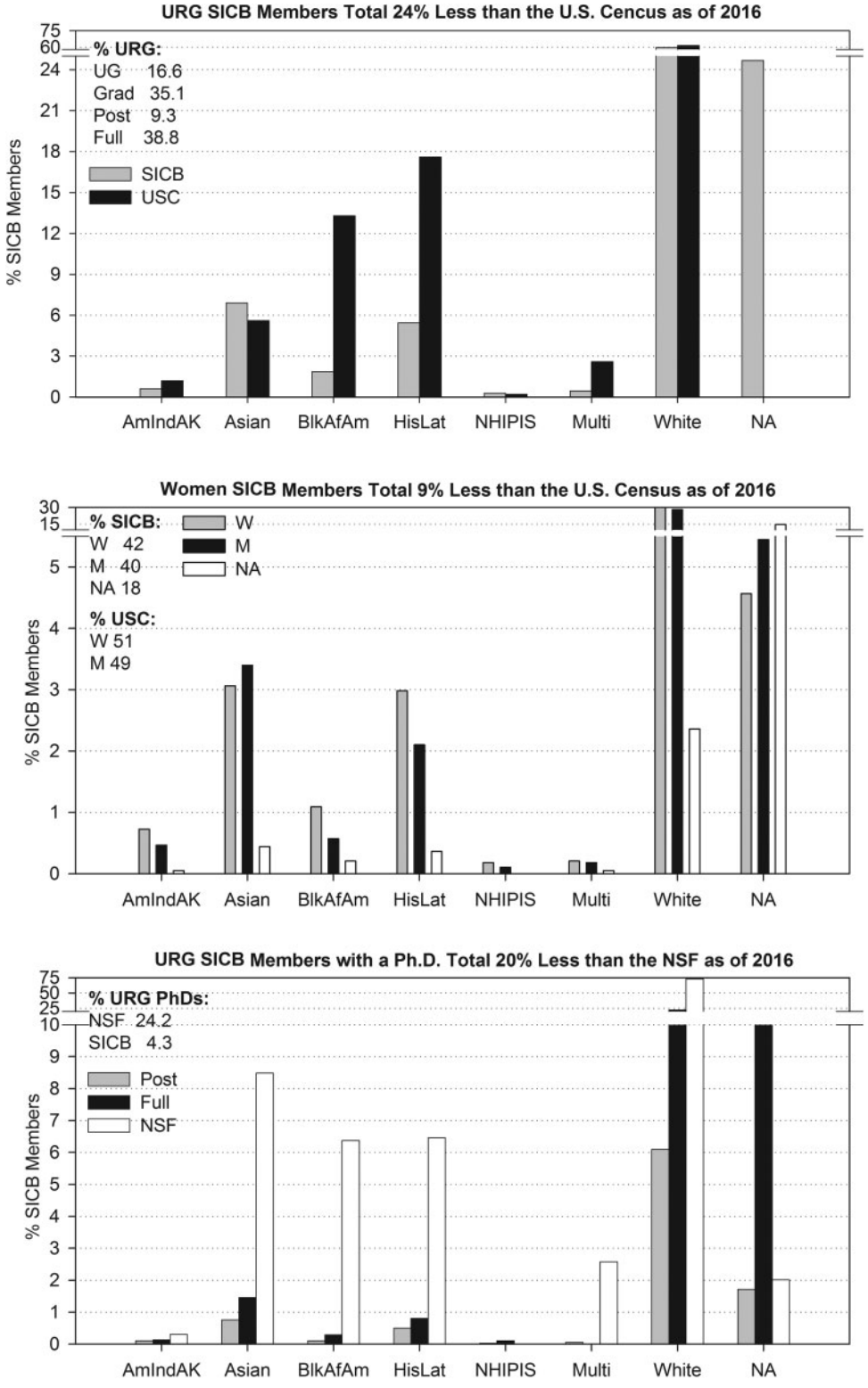


Fig. 1 Disparity in SICB membership relative to the US Census and National Science Foundation (NSF) PhD holders by ethnicity, gender and member level. SICB is comprised of 17% URG members, 4.3% of which hold Doctoral degrees, 42% women members and 8.2% are from URGs. Total 2016 membership is 3855. URG ethnicities: AmInAK, American Indian and Alaska Natives; BlkAfAm, Black and African Americans; HisLat, Hispanic and Latino; Multi, more than one ethnicity; NHIPI, Native Hawaiian and Pacific Islanders. Member level: Full, faculty; Grad, graduate; Post, postdoctorate; UG, undergraduate; Gender: F, female; M, male. NA, not answered. Census: NSF, 2014 NSF census of PhD holders; USC, 2015 US population census.

Table 1 Objectives of the Broadening Participation Committee

Objectives	Outcomes
1 + 2) Initiate a URG cohort and community that will be sustained throughout the career at all levels of membership. Addresses BEST #3–5.	Continue support for attending annual meetings, offer social events that bridge new and loyal URG members.
3) Provide leadership and professional training that sustains beyond their involvement with SICB. Addresses BEST #1, 6–7.	Offer workshops in best research practices such as grant writing, time management, leadership, funding opportunities.
4) Recruit members and promote the society to other organizations serving URG in the biological sciences relevant to the interests of the society. Addresses BEST #2–3.	Individual members who attend meetings such as AISES, SACNAS, MARC, AGEP and McNair can promote the benefits of being an active and diverse SICB member.
5) Determine whether the BPC initiatives are increasing diversity within the society and beyond. Addresses BEST #8.	Continually assess each aspect of the program, revise and redirect (if needed) the goals and objectives of the program.

Notes: AISES, American Indians in Science and Engineering; SACNAS, Society for the Advancement of Chicanos and Native Americans in Science; MARC, Maximizing Access to Research Careers; and AGEP, Alliances for Graduate Education and the Professoriate.

which SICB members are involved and 5) have measurable outcomes that can be used for assessment of BPC goals. Positive outcomes will drive the direction and evolution of future BPC objectives (see Table 1 for current objectives). We will maintain or enhance those activities that we find are successful and modify those that are not working until the percentage of URG members mirrors that of the US population census.

The current BPC goals align with prior key objectives that were identified as most successful in recruiting and retaining URG in other professional societies and in STEM fields (Wilson and Haynes 2002; Pandya et al. 2007; Payton et al. 2012).

SICB BPC activities and assessment

Demography of SICB

SICB URG member gains was measured by implementing a mechanism to collect and assess self-identification of ethnicity, gender and disability (added in 2011) on annual membership (2010–2016) and meeting registration forms (2009–2016). This was a critical step in determining the ethnic, race, gender and disability makeup of our membership compared with 2015 US Census and NSF Doctoral Degree-Holder demographics (NSF 2014; US Census 2015). As of the 2010 annual conference, SICB had 2373 members, of whom 11.0% were from URGs, 49.3% were white (39.7% did not self-identify ethnicity), with 32% women members (30% left gender blank). As of the 2016 annual conference, SICB membership increased to 3855 members, an increase of 62% from 2010 (Fig. 1). URG members increased to 16.2%, with an increase in white members to 59.8% (Fig. 2). Women members increased to 42%, while men members increased by 2% (Fig. 2). Thus, there was a sharp decrease in the percentage of members that failed to self-identify ethnicity (down to 24.7%) and gender (down to 18.1%; Fig. 2). Associated with the 15.0% increase in self-identification is a 10.5% increase in white and a 5.2% increase in URG members (Fig. 2). Even with these remarkable gains, URG membership within the society is only 16.2%, essentially half the 37.9% reported in the 2015 US Census (US Census 2015). SICB members reporting a disability is 1.5% compared with 8.5% of Doctoral Scientists in biological, agricultural and environmental life sciences (ages 16–64 years) of the general population in the United States (NSF 2014; US Census 2015).

Comparing the current membership of SICB to that of the 2015 US Population Census indicates that SICB is doing well attracting some URG groups (Fig. 1). Asians and Native Hawaiian/Pacific Islanders are the only URGs currently in SICB where the percent of members exceeds that of the population census (by 23.2% and 42.7%, respectively) (US Census 2015). Hispanic/Latino and American Indian/Alaskan Natives members together comprise less than or half that of the US population (31.0% and 51.9%, respectively, SICB/US%), with Black/African Americans members totaling only 14.0% of the BlkAfAm US population (SICB/US%). In 2016, SICB added a new category called "multiple ethnicities" to which 0.44% of members selected in lieu of selecting an ethnicity. This made it easier for some members with multiple ethnicities to self-identify (more than one group can be selected); however, this decreases the precision of our assessment. The percentage of white SICB members is slightly less than that of the US Census (97%, SICB/US%). Women members number slightly more than men, 42.3% and 40.1%, respectively, which are proportionately similar to the US Census (50.8% women, 49.2% men). However, unless those 18.1% of

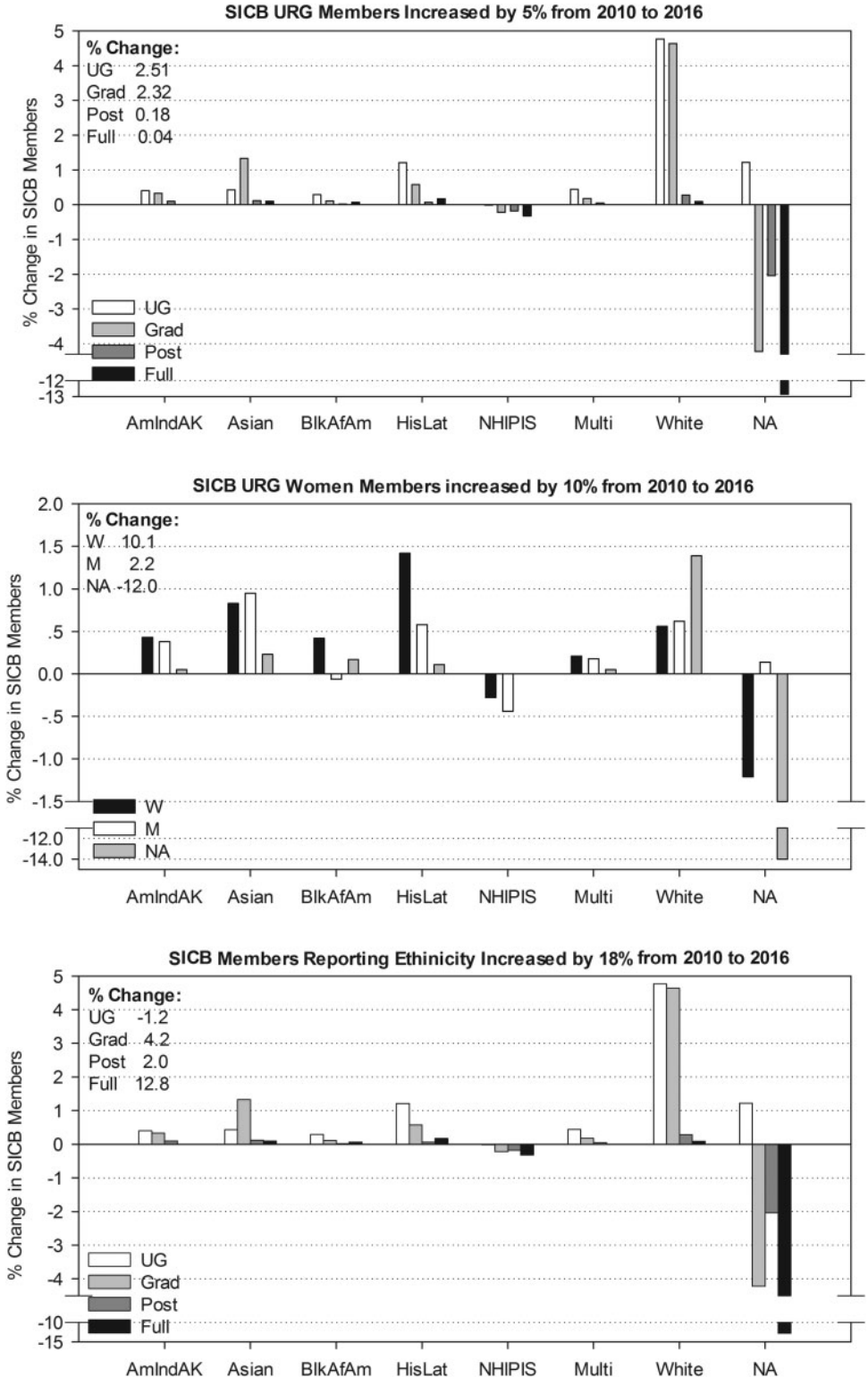


Fig. 2 Effects of BPC activities on SICB membership from 2010 to 2016. URG members increased from 11% to 16%, women members increased from 32% to 42% and member self-identification increased from 60% to 75%. Abbreviations as in Fig. 1.

members that leave gender blank self-identify, it will be unknown whether the gender ratio in SICB is truly equal to that of the US Census (US Census 2015).

SICB Membership consists of doctorate holding members that are Full (35% including 3.6% emeritus) and Postdoctoral (9.3%), with students comprising about half of the members: graduate (35%) and

undergraduate (16.7%) (Fig. 1). Interestingly, most of the emeritus and nearly one-third of full members fail to self-identify ethnicity (79.3% and 28.4%, respectively) compared with postdocs (18.3%), graduate students (16.4%) and high school students (14.3%). On the other hand, undergraduate students (25.4%) fail to self-identify nearly as often as full members. SICB is doing well at attracting graduate students and full members, but is only retaining approximately 26.3% of the graduate student members as postdoctoral members. This may be due to the lack of support for postdoctoral members to attend meetings. Attracting more postdoctoral researchers, who can benefit from professional workshops and networking at the socials could lead to increased SICB members once they obtain faculty positions and bring their postdoctoral researchers and students to the annual meeting. Sharing data about societal composition is a valuable resource for all professional societies to measure their efforts in the recruitment and retention of URG (Freehill and Ivie 2013). However, we were unable to find other scientific societies with similar membership data to compare our data too.

Relating the percentage of SICB members with Doctorates to the NSF Census of Doctoral Holders may be the more appropriate standard with which to compare the demography of a scientific society. Only 4.4% of URG SICB members hold a doctorate degree compared with 21.6% URG Doctoral Scientists reported in the 2014 Census of Doctoral Scientists in biological, agricultural and environmental life sciences in the United States (NSF 2014). SICB Full members are comprised largely of faculty in the academy, thus this may indicate a lack of successful recruitment of URG to the academy, or low URG recruitment to professional societies. URG SICB members with doctorates are fewer than NSF census Doctorate Holders in all ethnic categories: Hispanic/Latino with Doctorates (1.3%) in SICB compared with NSF Census Doctorate holders (6.5%); Asians with PhDs (2.2%) in SICB compared with NSF Census Doctorate holders (8.5%); Black/African Americans with PhDs (0.42%) in SICB compared with NSF Census Doctorate holders (6.4%) (NSF 2014). American Indian/Alaskan Native SICB members with Doctorates (9) are most similar to NSF census Doctoral Holders (0.23% versus 0.30%, respectively) because their populations are relatively low nationally. White SICB members with Doctorates comprise a third that of NSF Doctoral holders (29.3% versus 73.0%, respectively; NSF 2014).

Table 2 Funded travel award demographics from 2011 to 2016 (176 in total)

Ethnicity	Percent	Level and gender	Percent
Hispanic/Latino	55.9	Asst. Professors	7.3
Asian	8.5	Postdoctorates	20.9
Black/African American	13.0	PhD Students	28.8
American/Alaskan Indian	4.0	MS Students	15.8
Native Hawaiian/Pacific Islanders	5.1	UG Students	26.0
White women	13.6	Females	71.8
		Males	32.8

With respect to leadership, SICB has had only nine women and no minority presidents since its inception in 1890, which means that 92% of past presidential terms have been white men (117 terms). In addition, few URG members are holding leadership or divisional positions within SICB. The reason for this lack of leadership may be partly due to the low number of URG SICB members with Doctorates, particularly at the full professor level. Having URG member representation on the executive committee would provide role models within the society, and present a collective societal voice representing diversity and inclusion for all scientists.

The Travel Award Program started with the 2011 annual meeting and has been successful in other scientific societies as well as at SICB (Wilson and Haynes 2002). URG Members from all levels can apply for up to $500 to support travel to the annual meeting. As a mechanism to guide future efforts of the BPC to broaden participation within SICB, applicants are asked to state their career goals, describe two challenges to being a member from an URG in science and suggest workshop topics for the next annual meeting. The BPC was able to fund 86.6% of applicants in 2011, 53.6% of applicants in 2012, 91.6% of applicants in 2013, 92.5% of applicants in 2014, 58% of applicants in 2015 and 33% of applicants in 2016. Hispanic/Latino members received slightly more than half of the Travel Awards, followed by White Women and Black/African American members (Table 2). In 2011, more members were supported at a smaller amount than requested in order to fund most of the applicants. Many of the travel fellows verbally told BPC members that they would not have been able to attend without this funding. In 2012, most members requested the maximum amount stating that they would unlikely attend without full funding. Thus, fewer members were funded but with a higher

Table 3 Professional development workshops from 2011 to 2014

Year	Name, number of attendees	Hosts
2011	Balancing Life and an Academic Career	Greg Florant,[a,b] Nora Espinosa[a,b]
2011	Issues facing new faculty	Denise Dearing,[b] Peggy Biga,[b] Hannah Carey, Michele Nishiguchi,[a,b] Scott McWilliams
2012	Science is a Two-way street: Mentorship and the Mentee	Michele Nishiguchi,[a,b] Billie Swalla (President Elect), Cheryl Wilga[a,b]
2012	Demystifying the Grant Application Process	Cheryl Wilga,[a,b] Michele Elekonich and Bill Zamer (NSF Program Directors)
2013	Effective presentations skills	Manny Azzizi,[a] Patricia Hernandez,[a,b] Andrew Clark[a]
2013	How to negotiate your first job	Gregory Florant,[a,b] Billie Swalla (President)
2014	Recruitment strategies to obtain a diverse and thriving lab and department	Rebecca Calisi-Rodriguez,[a] Michele Nishiguchi,[a,b] Cheryl Wilga[a,b]
2014	Writing grants and manuscripts in a timely manner	Heather Bleakley, Brian Tsukimura[a,b] (Past Program Officer), Michele Nishiguchi[1,2]
2015	The academic juggling trick: how to effectively manage your time during the professoriate	Michele Nishiguchi[a,b]
2015	Don't be such a scientist, part II: How to give dynamic and informative presentations	Jake Socha (SICB Public Affairs Committee)
2016	Integrate diversity awareness into science institutions	Kendra Greenlee,[a,b] Michele Nishiguchi[a,b]

[a]URG member.
[b]BPC member.

level of support. In 2013, the deadline was moved earlier in the year to allow applicants to receive notification of the award before the registration deadline. Applicants requested the earlier deadline so they would know whether they had the funds to attend the meeting before registering for the meeting, however several members missed the earlier deadline. In 2014, BPC members Cheryl Wilga, Michele Nishiguchi and Brian Tsukimura were awarded an NSF Conference Grant for $25,000 to fund two SICB Broadening Participation workshops, URG workshop panelists and URG members at the annual meeting (IOS-1362663). As a result, 64 additional members were funded (92.5% of applicants). In 2014–2016, postdoc and junior faculty URG members were given priority to BPC travel funds in an attempt to increase the low member attendance at those levels. Overall Travel Awards were fairly equally spread out among undergraduate, doctoral and postdoctoral members, with a smaller percentage going to masters student and assistant professor members. Two-thirds of the Travel Award recipients were women.

The BPC offers two Professional Development Workshops at each annual meeting starting with the 2011 meeting. This is a main feature of broadening participation efforts in other scientific societies (Wilson and Haynes 2002). Workshops are chosen from the most requested topics suggested by the Travel Award Fellows. All SICB members are invited, and every workshop thus far has been successful with a mean of 74 members attending (range 30–100) (Table 3). One BPC workshop focuses on professional development for graduate students and the other focuses on faculty and postdocs. Of the 26 workshop hosts from 2011 to 2016: 16 were from URGs; 18 were women; 16 were Full Professors; 2 were Associate Professors; 6 were Assistant Professors; 2 were NSF Program Officers; 6 were current or past Chairs of the BPC; 9 were BPC members and 3 were SICB Executive Committee members (President and Program Officer) (Table 3).

The pre-meeting ''Meet and Greet'' Social is hosted by the current Chair of the BPC on the first day before the plenary lecture and was initiated in 2012. This social brings together travel award fellows and members of the BPC for networking and to establish a cohort that will eventually build community within SICB and increase retention and form lasting post-meeting relationships. The pre-meeting strategy is to create a cohort of members at each meeting that can reconvene throughout the meeting to share thoughts and impressions about the meeting. Appetizers and soft drinks are provided by SICB funds in an informal setting with an attendance of approximately 30–50 members each year.

The Broadening Participation Diversity Social that started with the 2011 meeting has also triumphed in

increasing awareness of the committee and its activities. Here the BPC provides a spread of appetizers, invites guest speakers (BPC members, NSF Program Officers, Past and Current SICB Presidents and SICB Executive Committee Members), recognizes the BPC Travel Fellows, presents the travel awards and provides a comfortable friendly atmosphere for all members to mingle and chat. Attendance started in 2011 at around 100 members, with at least 300 in 2013, and approximately 200 members in 2014–2016. Our hope is that members from URGs feel more comfortable interacting with current and past executive committee and BPC members, NSF program officers, and other members in a smaller, intimate atmosphere and therefore become more involved in the society. This also provides past and future cohorts with a venue for informal networking with leaders in their fields and within the Society.

Impact of BPC activities

Over the past 5 years, the data show an increase in URG members (up by 5.2%), a decrease in members who do not report ethnicity and gender (down by 15% and 12%), and an increase in the number of members attending BPC activities (up to 33% by 2016) (Fig. 3). The striking decrease in the number of members who do not report ethnicity, especially over the last two years, suggests an increased awareness throughout SICB of the benefits of having a diverse membership. We are particularly pleased to see that postdoctoral member attendance is also steadily increasing. BPC efforts have succeeded in increasing diversity within SICB membership as well as those attending annual meetings as indicated by the steady increase in URG members and BPC activity attendance.

Travel awards have increased participation and enhanced attendance of URGs. Assessment of the BPC Travel Award program is very encouraging. Over the past 4 years, the BPC was able to fund 63.6% of the applicants, with 5% withdrawing their applications due to other funding being secured or inability to attend the meeting (Table 4). Funding for travel to annual SICB meetings is critical because 33% of the unfunded applicants did not attend the annual meeting (Table 4). Funding is also critical for continuing membership. Of those applicants that were funded (176 total), 38% remained in SICB as of 2016, with 41% leaving after 1 year of membership and only 14% leaving after 2 years of membership (Table 4). Nearly half of the awardees that leave SICB after at least 1 year are undergraduate students (44%), with graduate students the next largest group (33%). Thus, it appears that most Travel Award applicants decide after two meetings whether SICB fits their needs for a professional scientific society.

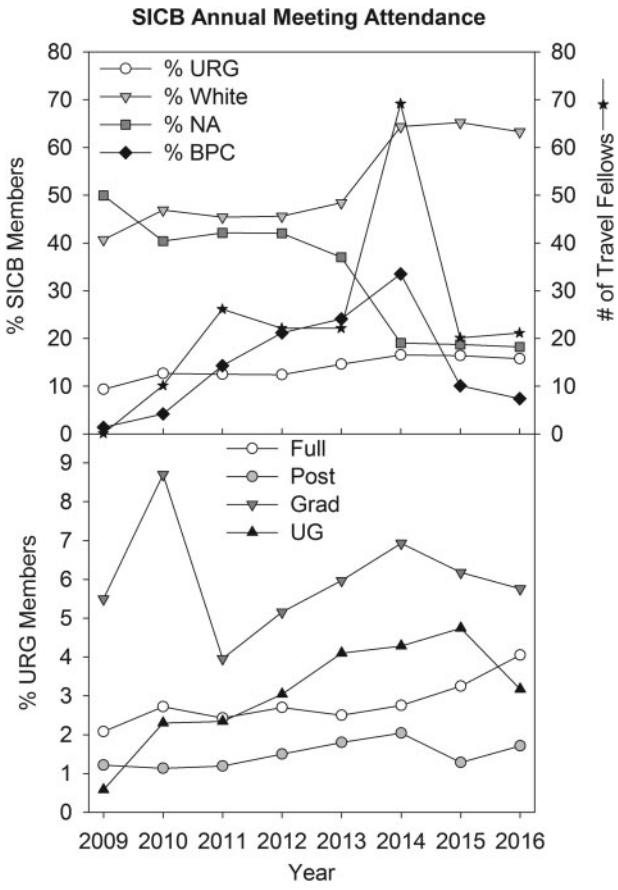


Fig. 3 Effects of BPC activities on SICB member attendance at annual meetings. The top plot shows the percent of all members who attended the annual meeting by ethnicity, failed to self-identify and attended BPC activities. The number of URG members funded by travel awards is shown on the right axis (black asterisk). Note the sharp decline in the percent of members who fail to self-identify ethnicity (NA, gray boxes). Also note the steady increase in URG attendance with increased funding (open circles) and BPC professional development and social activities (black diamonds). The increase in 2014 attendance is due to the increase in BPC URG travel awards funded by a NSF meeting award. The bottom plot shows the demographics of URG members by level who attended the annual meeting. Note the steady increase in the percent of full and postdoctoral members (open and gray circles, respectively).

To maintain or increase retention, it is imperative that a sense of community among the participants be developed (Hassoun and Bana 2001; Peckham et al. 2007; Jones et al. 2008; Koenig 2009; Xu and Martin 2011). Community can be developed through interactional engagement and social activities, such as pre-meeting and intra-meeting socials (Peckham et al. 2007; Jones et al. 2008; Xu and Martin 2011).

Table 4 Assessment of travel award applicants from 2010 to 2016

Travel award applicants = 176 total	%
Unfunded applicants of total	31.0
Unfunded applicants who also did not attend annual meeting	33.3
Withdrawn applicants of total	9.1
Funded applicants of total	63.6
Funded applicant status = 112 total	% funded
Remained SICB members as of 2016	37.9
Left SICB 1 year after funding	40.8
Left SICB 2 years after funding	14.4
Left SICB 3 years after funding	4.0
Left SICB 4 years after funding	2.9
Left SICB 5 years after funding	0.0
Received awards 2 years	10.5
Received awards 3 years	0.7
Received awards 4 years	0.9

URG members with increased interaction with executive committee and divisional leaders can develop a sense of community within SICB, especially if they feel that they are welcomed by the SICB's membership (Informal Professional Networks; Xu and Martin 2011). Mentoring, on informal individual and workshop scales, from peers and SICB leadership, can lower perceived barriers about engaging with senior members (Pandya et al. 2007; Koenig 2009; Tapia 2009; Payton et al. 2012; Wilson et al. 2012). In particular, mentoring early in one's career is critical to enhancing professional success (Jones 2014).

Traditional presentations of the academy, and hence the professional society, are often culturally neutral ignoring participant perception or understanding of basic academy operations (Peckham et al. 2007; Jones et al. 2008). Shared experiences can often bring incongruent backgrounds together and initiate forming a sense of community. Our travel awardees form a natural cohort, which we bring together several times throughout the meeting at BPC socials and workshops so that they might find each other among the crowds, and to find how the meeting itself is a common experience.

We have increased retention through a supportive community formed through the BPC Travel Fellows, workshops and social programs. Continuing and bridging support to the next level of membership can be provided informally by SICB members, but also through BPC workshops. The Travel Fellows Program form a cohort facilitated by the socials and workshops that we hope will stimulate sustained interaction long after the annual meeting. The BPC Pre-meeting and Diversity Socials, open to the entire society, are informal and friendly places where these networks can easily be formed, increasing the potential for URG professional networking and mentoring (Xu and Martin 2011). The very successful and well attended BPC Diversity Social and workshops are already building community within the society and indicates buy in by member attendance.

SICB has already made a significant investment in broadening participation, not only financially, but also with a commitment by its members to increase URG diversity. Members are beginning to realize that increasing diversity will not only impact the Society's ability to successfully facilitate different approaches to tackling problems within integrative biology, but will also help positively impact larger issues that develop throughout science and technology fields. A team composed of diverse members generates a greater breadth of solutions (i.e., "Grand Challenges in Organismal Biology—The need for synthesis" by Padilla et al. 2014). In order to facilitate and build a long-lasting community, SICB has provided the springboard in the BPC to initiate the welcoming of URGs into the society by striving for the goals stated in this paper. The SICB has also committed to a long-lasting and sustainable program that will recapitulate benefits from which all members of the society will gain, especially once SICB demographics match that of the US Census. SICB is a leader among long-lived scientific societies, and by succeeding in the increased awareness and participation of URGs, we will create a community of leaders who can bring their values, ideas and knowledge to improve broader scientific challenges that are of greater importance in today's world.

Acknowledgments

We thank the Executive Committee, Division and Program Officers, Members and Staff in the Society for Integrative and Comparative Biology and Burke Inc. for fully and enthusiastically supporting the BPC's efforts to broaden participation.

Funding

This work was supported by the Society for Integrative and Comparative Biology and by the National Science Foundation [IOS-1362663]. Meeting award to the authors for broadening participation at the annual society meeting.

References

Ely RJ, Thomas DA. 2001. Cultural diversity at work: the effects of diversity perspectives on work group processes and outcomes. Admin Sci Quart 46:229–73.

Freehill LM, Ivie R. 2013. Increasing the visibility on women of color in academic science and engineering: professional society data. New Direct High Educ 163:7–12.

Gardner SK. 2013. The challenges of first-generation doctoral students. New Direct High Educ 163:43–54.

Goode TD. 2004. National center for cultural competence. Georgetown University Center for Child and Human Development, University Center for Excellence in Developmental Disabilities.

Gurin P, Nagda RA, Lopez GE. 2004. The benefits of diversity in education for democratic citizenship. J Soc Issues 60:17–37.

Hassoun S, Bana S. 2001. Practices for recruiting and retaining graduate women students in computer science and engineering, Microelectronic Systems Education, 2001. Proceedings. 2001 International Conference on Microelectric Systems Education, pp. 106–107.

Holly KA. 2013. How diversity influences knowledge, identity, and doctoral education. New Direct High Educ 163:99–105.

Jones KW, Kristof D, Jenkins LC, Ramsey J, Patrick D, Burnham S, Turner IL. 2008. Collaborative technologies: Cognitive apprenticeship, training, and education. Collaborative Technologies and Systems, 2008. International Symposium Microelectric Systems Education, pp. 452–459.

Jones J. 2014. The Chronicle of Higher Education. Clogged Pipelines. March 7, Supplement pp. 45–48.

Kendall FE. 2011. Multicultural education program—evolving community at Cal: equity, inclusion, and diversity, University of California, Berkeley.

Koenig R. 2009. Minority retention rates in science are sore spot for most universities. Science 324:1386–7.

McLeod PL, Lobel S, Cox T. 1996. Ethnic diversity and creativity in small groups. Small Group Res 27:827–47.

National Science Foundation (NSF). 2014. National Center for Science and Engineering Statistics, Survey of Doctorate Recipients, 2013. https://nsf.gov/statistics/2016/nsf16300/digest/.

Padilla DK, Daniel TL, Dickinson P, Grunbaum D, Hayashi C, Manahan DT, Marden JH, Swalla BJ, Tsukimura B. 2014. Addressing grand challenges in organismal biology—the need for synthesis. BioScience 64:1178–87.

Pandya RE, Henderson S, Anthes RA, Johnson RM. 2007. BEST Practices for Broadening Participation in the Geosciences: Strategies from the UCAR Significant Opportunities in Atmospheric Research and Science (SOARS) Program. J Geo Educ 55:500–6.

Payton FC, Suarez-Brown TL, Lamar CS. 2012. Applying IRSS theory: The Clark Atlanta University exemplar. J Innov Educ 10:495–513.

Peckham J, Stephenson PD, Harlow LL, Stuart DA, Silver S, Mederer H. 2007. Broadening participation in computing: issues and challenges 2007. ACM SIGCSE Bulletin 39:9–13.

Schmidt P. 2010. Graduate programs grow less diverse without racial preferences, research suggests. The Chronicle of Higher Education. May 14, 2012 (http://chronicle.com/article/Graduate-Programs-Become-Much/125459/).

Smith DG. 1991. The challenge of diversity: alienation in the academy and its implications for faculty. J Excel College Teach 2:129–37.

Tapia R. 2009. Minority students and research universities: How to overcome the 'mismatch'. The Chronicle of Higher Education 55:A72.

US Census. 2015. Population Estimates Program. Quick Facts. United States. https://www.census.gov/quickfacts/table/PST045216/00.

Wilson DJ, Haynes JK. 2002. ASCB minorities affairs committee goals: ''Strengthening the chain of success''. Cell Biol Educ 1:105–6.

Wilson ZS, Holmes L, deGravelles K, Sylvain MR, Batiste L, Johnson M, McGuire SY, Pang SS, Warner IM. 2012. Hierarchical Mentoring: A transformative strategy for improving diversity and retention in undergraduate STEM disciplines. J Sci Educ Technol 21:148–56.

Xu Y, Martin C. 2011. Gender differences in STEM disciplines: from the aspects of informal professional networking and faculty career development. Gender Issues 28: 134–54.

TITLE: Phylogeographic patterns in the Philippine archipelago influence symbiont diversity in the bobtail squid-*Vibrio* mutualism

AUTHORS: [1]Randy Lane Coryell, [1]Kira Emily Turnham, [2]Evelyn Grace de Jesus Ayson, [2]Celia Lavilla-Pltogo, [3]Angel C. Alcala, [4]Filippina Sotto, [5]Benjamin Gonzales, and [1]Michele Kiyoko Nishiguchi

POSTAL ADDRESSES:

1. New Mexico State University, Department of Biology, MSC 3AF, P.O. Box 30001, Las Cruces NM 88003-8001, USA
2. Southeast Asian Fisheries Development Center (SEAFDEC), Buyu-an, Tigbauan, 5021 Iloilo, Philippines
3. Silliman University, 1 Hibbard Avenue, Dumaguete City, 6200 Negros Oriental, Philippines
4. University of San Carlos, Governor M. Cuenco Ave., Cebu City, 6000 Cebu, Philippines
5. Western Philippine University, San Juan, Aborlan, Palawan, 5302, Philippines

KEYWORDS: *Vibrio*, Squid, Symbiosis, Phylogeography,

CORRESPONDING AUTHOR: Michele K. Nishiguchi, Department of Biology, New Mexico State University, P.O. Box 30001, MSC 3AF, Foster Hall- 1200 S. Horseshoe Dr., Las Cruces, NM 88003-8001, USA

email: nish@nmsu.edu

RUNNING TITLE: Philippine squid-*Vibrio* phylogeography

ABSTRACT

Marine microbes encounter a myriad of biotic and abiotic factors that can impact fitness by limiting their range and capacity to move between habitats. This is especially true for environmentally transmitted bacteria that cycle between their hosts and the surrounding habitat. Since geologic history, biogeography, and other factors such as water temperature, salinity, and physical barriers can inhibit bacterial movement to novel environments, we chose to examine the genetic architecture of *Euprymna albatrossae* (Mollusca: Cephalopoda) and their *Vibrio fischeri* symbionts in the Philippine archipelago using a combined phylogeographic approach. Fourteen separate sites in the Philippine islands were examined using haplotype estimates that were examined via nested clade analysis to determine the relationship between *E. albatrossae* and *V. fischeri* populations and their geographic location. Identical analyses of molecular variance (AMOVA) were used to estimate variation within and between populations for host and symbiont genetic data. Host animals demonstrated a significant amount of variation within island groups, while symbiont variation was found within individual populations. Nested clade phylogenetic analysis revealed that hosts and symbionts may have colonized this area at different times, with a sudden change in habitat. Additionally, host data indicate restricted gene flow, whereas symbionts show range expansion, followed by periodic restriction to genetic flow. These differences between host and symbiont networks indicate that factors “outside the squid” influence distribution of Philippine *V. fischeri.* Our results shed light on how geography and changing environmental factors can impact marine symbiotic associations at both local and global scales.

INTRODUCTION

The dispersal of marine species across suitable habitats can be affected by physical barriers (temperature, distances across oceans, island formations) as well as life history strategies (e.g. dispersal method of larvae, adult motility; (Kool, Paris, Barber, & Cowen, 2011). Biogeographical barriers, as reported by floral and faunal separations, occur worldwide and provide an opportunity to study how physical barriers coupled with other abiotic factors may be affecting species dispersal and ultimately distribution (Lohman et al., 2011; Tonon et al., 2015). Analysis of population structure and physical orientation of the distribution of taxa across these barriers has given us clues to the factors that fragment available habitat (Esselstyn et al., 2010). Although previous work has provided evidence for several causes for speciation among closely related populations in areas where distinct barriers exist, there is less known about species that coexist with one another, and whether rules that govern distribution patterns via allopatric speciation influence such associations (Hellberg, Burton, Neigel, & Palumbi, 2002; S. R. Palumbi, 1994).

One region that has been studied extensively for its unique patterns of biogeography and geologic history is the Indo-Pacific barrier (IPB), which was created by the uprising of the Indonesian archipelago separating the Indian and Pacific oceans (Gaither, Toonen, Robertson, Planes, & Bowen, 2010). Interestingly, dispersal mechanisms and rapid adult motility have allowed certain taxa in the region to cross the IPB due to various dispersal strategies and larval residence time prior to metamorphosis compared to other taxa which are geographically restricted (Horne, 2014; Liu, Chang, Borsa, Chen, & Dai, 2014; Sorenson, Allen, Erdmann, Dai, & Liu, 2014). As part of the

IPB, the Philippine island archipelago is a “hotspot” for species diversity and endism and has warranted investigation of the distribution of taxa across the region (Roberts et al., 2002). In the Philippines, current research has focused on the phylogeographic distribution of some fishes, bent-toed geckoes, as well as bivalves across established biogeographical margins that limit some other terrestrial and marine taxa (Carpenter & Springer, 2005; Esselstyn et al., 2010; Gaither & Rocha, 2013; Huxley, 1868; Lemer et al., 2016; Siler, Oaks, Esselstyn, Diesmos, & Brown, 2010; Wallace, 1860, 1863). Local analysis of the distribution and connectivity of some marine taxa across the Philippines has also been investigated in western populations of the sea star *Linckia laevigata* and the giant clam *Tridacna crocea* near the island of Palawan, as well in the western portion of the Central Visayas (Alcazar & Kochzius, 2016; Juinio-Menez, Magsino, Ravago-Gotanco, & Yu, 2003; Magsino, Ravago, & Juinio-Menez, 2002; Ravago-Gotanco, Magsino, & Juinio-Menez, 2007). Interestingly, very few studies have examined the connectivity of populations across the whole of the Philippine archipelago, and what impact physical factors, life history, and geographical barriers have on the distribution of mutualist partners. This has created a void in the knowledge of how local assemblages of mutualist associations are impacted by geography in this unique area. Therefore, there is a need to better understand whether the physical barriers in this area have shaped the distribution of coexisting marine organisms, and to determine what impact these physical factors have on species interactions, particularly between microbes and their eukaryotic hosts.

Across the globe, sepiolid squids (Cephalopoda: Sepiolidae) form mutualistic associations with bioluminescent bacteria from the genera *Vibrio* and *Photobacterium* (γ-

Proteobacteria: Vibrionaceae (Herring, 1977). *Vibrio* bacteria are housed within a specialized internal organ called the light organ (LO), where the host provides a nutrient rich habitat for the symbiont, and in return *Vibrio* bacteria provide luminescence to the squid to be used in a behavior termed counter-illumination (Jones & Nishiguchi, 2004). Squid hosts use the *Vibrio* produced light to reduce their silhouette during the evening, which enhances their survivability and predation success (McFall-Ngai, Heath-Heckman, Gillette, Peyer, & Harvie, 2012). After each nightly foraging session, approximately 95% of the *Vibrio* bacteria are vented out into the surrounding seawater, seeding the local area with symbiotically viable Vibrios (Boettcher, Ruby, & McFall-Ngai, 1996). Local cycling of symbiotic *V. fischeri* exposes these bacteria to a wide range of abiotic and biotic factors outside the host that can affect their fitness and ability to infect new hosts. This also allows for symbiotically competent free-living bacteria to migrate to new host habitats, where they can invade and colonize different populations of sepiolids (Nyholm, 2004; Nyholm & Nishiguchi, 2008; Nyholm, Stabb, Ruby, & McFall-Ngai, 2000).

Earlier work on sepiolid squids has focused on the influence of geographic distance on symbiont prevalence and genotype in both sympatric and allopatric populations (Jones, Lopez, Huttenburg, & Nishiguchi, 2006; Kimbell, McFall-Ngai, & Roderick, 2002; Zamborsky & Nishiguchi, 2011). Allopatric and sympatric populations for both squids and *Vibrio* bacteria show distinct population breaks that are not necessarily driven by host specificity. Additionally, host mediated factors along with abiotic variables such as water temperature and salinity have been known to shape these mutualist assemblages (McFall-Ngai, 2014; McFall-Ngai et al., 2012; Nishiguchi, 2000; Soto, Gutierrez, Remmenga, & Nishiguchi, 2009). Collectively, either genomic comparison of

115 closely related populations (Bongrand et al., 2016) or haplotype comparisons of
116 allopatric populations of Indo-west Pacific squid and their vibrio symbionts (Jones et al.,
117 2006) do not address the connectivity of populations across physical and
118 biogeographical barriers like those in the Philippines or across the IPB. Therefore, we
119 examined the genetic architecture of *Euprymna albatrossae* (Cephalopoda: Sepiolidae)
120 and their *V. fischeri* symbionts in the Philippine archipelago using a combined
121 phylogeographic approach to determine whether host specificity or geographic location
122 influence the distribution of symbiotic Vibrios in this region. The unique geographic origin
123 of the Philippines, its proximity to deeper and colder water, as well as currents that move
124 through the area allows for investigation of what roles geography and host specificity
125 have in the distribution of mutualistic associations.
126
127 **Methods**
128 ***Specimen collection and bacterial isolation***
129 Squids were collected in the months May, June, July, and August during the
130 years 2010, 2012, 2013, and 2015 at eleven different sites around the Philippine islands
131 (Fig. 1, Table 1). Adult squid, (~5-9 cm in length) were acquired either by dip or seine
132 net. Captured squids were brought back to the laboratory and placed on ice to
133 anesthetize them prior to dissection. Host light organs were subsequently removed via
134 ventral dissection and homogenized to plate on seawater tryptone agar plates (SWT;
135 0.5% tryptone, 0.3% yeast extract, 0.3% glycerol, 1.5% agar, and 70% seawater at 32
136 ppt) to isolate single colonies of *V. fischeri*. Plates used for light organ isolation were
137 made with local seawater from SEAFDEC, while all other plates were made with artificial

seawater containing a mixture of Instant ocean (21 g/L of seawater; Spectrum Brands, VA) and Marine Mix (7 g/L of seawater; Wiegandt GmbH, Germany). Squid tissues were preserved in 95% ethanol for fixation and subsequent DNA extraction for Sanger sequence analysis at New Mexico State University (NMSU).

Light organ homogenates were grown for 12 to 24 hours at 20°-28°C, after which 10-15 individual colonies from each plate were stab inoculated into vials containing SWT agar and sealed for transport back to NMSU. After transport, each sample was re-cultured on SWT agar plates at 28°C for 12-24h. Single colonies were isolated and cultured in liquid SWT at 28°C and shaken at 225 rpm for 12-18 hours in an Innova 43 shaking incubator (New Brunswick Scientific, NJ). Each overnight culture was sub cultured and allowed to reach log phase (2-3 hours at 28°C and 225 rpm), and the log phase cultures were used for DNA extraction and also frozen in 40% glycerol for storage at -80°C.

DNA extraction and amplification

E. albatrossae DNA was extracted by using approximately 25 mg of ethanol preserved tissue that was dissected from the gill or mantle of each squid. Dissected tissues were washed with 100 µL of nuclease free water to remove any residual ethanol. *E. albatrossae* DNA was extracted using the DNeasy© blood and tissue protocol for animal tissues (Qiagen, Valencia, CA). All genomic DNA extractions were visualized on a 1% agarose gel and quantified using a Nanodrop 9600 (Thermo Fischer Scientific, Waltham, MA). Total DNA extracted from each individual squid sample was used to amplify a 658-bp fragment of the cytochrome *c* oxidase subunit I (COI, Table 2; (Folmer,

Black, Hoeh, Lutz, & Vrijenhoek, 1994; Jones et al., 2006; Zamborsky & Nishiguchi, 2011). The cytochrome c oxidase subunit one gene has been shown to be highly conserved, at least at the amino acid level, across invertebrate taxa (Folmer et al., 1994; Jacobs & Grimes, 1986) and has been used extensively to elucidate population structure (Calderon, Garrabou, & Aurelle, 2006; Lessios, Kane, & Evolution, 2003; S. Palumbi, Grabowsky, Duda, Geyer, & Tachino, 1997).

Isolation of DNA from *V. fischeri* light organ isolates was completed using the Qiagen DNeasy© blood and tissue kit (Valencia, CA) gram-negative bacterial protocol. Approximately 2 X 10^9 cells were transferred from each log phase culture to the extraction tube for centrifugation. After, the remaining pellet was used for extraction using the Qiagen protocol. Purified *V. fischeri* DNA was visualized on a 1% agarose gel and quantified using a Nanodrop 9600 (Thermo Fischer Scientific, Waltham, MA). Isolated DNA extracted from each *V. fischeri* isolate was used to amplify a portion of the glyceraldehyde phosphate dehydrogenase (*gapA*) locus (~900 bp) by PCR, using previously described *Vibrio* specific primers (Table 2; (Jones et al., 2006; Nishiguchi & Nair, 2003). The *gapA* locus has been used reliably to estimate deep phylogenetic connections between bacterial families (Nelson, Whittam, & Selander, 1991) within the Vibrionaceae (Thompson, Gomez-Gil, Vasconcelos, & Sawabe, 2007) as well local population structure of mutualist *V. fischeri* (Jones et al., 2006; Nishiguchi & Nair, 2003).

Each PCR amplification reaction (25 µL) contained 2-20 ng of template DNA [0.08-0.8 ng/ µL], GoTaq DNA polymerase [0.05 U/ µL] (Promega, Fitchburg, WI), 5X GoTaq buffer [1x] (Promega, Fitchburg, WI), a 10 mM deoxynucleoside triphosphate mix [0.8 mM] of each nucleotide (Promega, Fitchburg, WI), and both forward and reverse

primers [0.5 μM each] (Table 2). All amplification reactions were run using a MJ Research Dyad Disciple thermocycler (Waltham, MA). Cycle conditions for each reaction are listed in Table 3. Amplicons were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and quantified using a Nanodrop 9600 (Thermo Fischer Scientific, Waltham, MA). Purified amplicons were presequenced using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) and amplified on an MJ Research Dyad Disciple thermocycler (Waltham, MA). Preseqencing samples were cleaned using 96-well Sephadex plates (Edge Biosystems, St. Louis, MO). Samples were sequenced at the NMSU Molecular Biology Sequencing facility using the Applied Biosystems 3130XL sequencer (Applied Biosystems, Foster City, CA). Sequences were assembled and aligned using GENEious (Biomatters Ltd, v7).

Haplotype networks, nested clade analysis, and molecular variance

Haplotype networks for squid and symbiont were generated using TCS v1.12 using statistical parsimony methods outlined by Templeton (Templeton & Sing, 1993). Nested clade analyses were performed using Templeton's nesting algorithm as implemented in GEODIS (Posada, Crandall, & Templeton, 2000). Analysis of molecular variance (AMOVA) was executed using the population genetics software platform ARLEQUIN (Excoffier & Lischer, 2010). Analyses were run for measures of within and among population variation along with a separate analysis assessing variation by island for both host and symbiont data. Concurrently, theta (σ), a base pair by base pair measure of polymorphism was calculated for each mutualist population at each sample site.

Results

Nested clade and molecular variance analysis of E. albatrossae

A total of 81 host COI sequences were used in the nested clade analyses of host genetic data, resulting in 43 distinct squid haplotypes (Genbank; MF379363 - MF379405). Host genetic data yielded three distinct unconnected haplotype networks, with one network containing only samples from the island of Palawan, while the other two networks exhibited introgression from the central island chain but no connection from the Palawan population (Fig. 2). Interestingly, host haplotype networks demonstrate little genetic connection between geographically separated populations. One host network (Clade 4-7, Fig. 2) has most members of this haplotype from the island of Panay with small contributions from populations found near the island of Negros, but no contributions from nearby Cebu populations. Another separate host network (Clade 4-1, Fig. 2) demonstrates genetic connection between Cebu, Negros, and a small contribution from Panay but also no connection to Palawan. The largest haplotype in clade 4-7 (Haplotype 14) is the result of equal contributions of genetic information from SEAFDEC, Santo Niño Sur, and Banate, all from the island of Panay. The dominant haplotype from clade 4-1 (Haplotype 5) has the largest contribution from populations sampled at Cordova and Magellan Bay from the island of Cebu, with a small contribution in this clade from SEAFDEC and Santo Niño Sur populations from the island of Panay. The third host network, clade 4-2, has no connection between Palawan host populations and hosts from the central island chain (Fig. 2).

Final nested clade analysis was performed with no detectable loops according to rules established by Templeton (Templeton & Sing, 1993). Nested clade phylogenetic analysis of host genetic data demonstrates that the null hypothesis of panmixia was rejected in four of the nested clades and for the total cladogram (Fig. 2). Inference from clade 2-3 indicates allopatric fragmentation involving populations from Cebu and Negros (Table 4; Fig.2). Clade 2-11 has restricted gene flow with isolation by distance involving populations from the island of Panay exclusively. Clade 3-1 also demonstrated restricted gene flow with isolation by distance, with clade 2-3 nested within and including genetic contributions from populations from all three central islands sampled (Table 4; Fig. 2). Clade 3-7, which includes subclade 2-11 as an interior clade (Table 4) inferred contiguous range expansion for populations. Total host cladogram inference was inconclusive due to the lack of connection between higher level clades (Table 4).

Analysis of molecular variance of host genetic data revealed that a significant portion of the variance was detected among islands and within populations (66.61%; 29.10%, Table 3). Some of the highest amounts of within-population genetic diversity, reported as theta, were observed at Dumaguete, Sibulan, and Siliman, which are all populations located from the island of Negros (0.1444, 0.0885, 0.0248; Table 1). Additionally, theta measures of populations at Parara, San Juan Barotec, and Santo Niño Sur from the island of Panay demonstrate significant within population diversity at these sites (0.0722, 0.0467, 0.0441; Table 1). Genetic diversity among populations near the island of Palawan was also observed to be a significant source of variation (0.0431, Table 1). The lowest amount of genetic diversity was detected at Cordova on the island

of Cebu, as well as at SEAFDEC and Banate from the island of Panay (0.00135, 0.0055, 0.0067; Table 1).

Nested clade and molecular variance analysis of V. fischeri

Conversely, symbiont genetic architecture in the Philippines displays a different pattern compared to their host squid. Successful initial colonization of juvenile light organs is accomplished by 1-3 strains that persist throughout the life of the animal (Wollenberg & Ruby, 2009). Any identical sequences, isolated from the same light organ were removed. Analysis of 181 symbiont *gapA* sequences yielded one contiguous network of 60 haplotypes (Genbank; MF379406 - MF379465). In contrast to host genetic architecture, symbiont populations are more connected compared to host populations. *Vibrio* genetic data produced a highly connected and diverse network showing genetic continuity between Palawan and the central island chain populations of symbionts (Figs. 3 & 4). Haplotypes 1, 3, 8, 57, and 58 contain representatives from each of the island populations sampled (Fig. 3). Major contributions from the western island of Palawan to haplotypes 8, 57 and 58 occur despite no host genetic introgression (Figs. 2 and 3). The largest haplotype (Haplotype 1) contains a significant number of Cebu haplotypes coupled with populations from Negros and Palawan. Each of the major haplotypes listed require a minimum of one base pair change, with the largest number of changes needed (6) to go from haplotype 1 to haplotype 8.

Contingency analyses of symbiotic *V. fischeri* nesting revealed significant evidence for restricted gene flow with isolation by distance in clades 2-1 and 2-8 (Table 5). Clade 2-1 exhibits restriction within the central island chain and connection between

small haplotypes found near Panay, Negros, and Cebu (Haplotypes 14, 15, and 16. Fig. 4.). Clade 2-8, which includes haplotypes 6, 8, and 9 grouped together with haplotypes 7 and 11 (Fig. 4) shows connection between central island populations and populations from Palawan. Inference from clade 3-5 indicates continuous range expansion of these populations. The grouping of clade 3-5 indicates a genetic connection between Panay and Dumaguete populations specifically from the island of Negros. Clade 4-2 also illustrates continuous range expansion and includes the subclades 2-1 and 2-8, which at the lower nesting level demonstrate restricted gene flow and isolation by distance (Table 5). Clade 4-2 also includes several singleton haplotypes that connect Palawan with the central island populations (Fig. 4). Total cladogram inference indicates, as in some of the lower level clades, restricted gene flow with isolation by distance (Table 5).

An Identical AMOVA analysis of symbiont genetic data revealed that a significant portion of the variance exhibited by these populations exists within and among populations (14.40%, 80.08%; Table 3). Base-pair by base-pair nucleotide diversity of host populations was highest in populations from Atabayan and Parara, Panay (0.0085, 0.0076; Table 1) and from the island of Negros (0.0074, 0.0061, 0.0050; Table 1) which are both in the central island chain. The lowest amount of genetic variation was observed at Magellan Bay, Macatan and Banate, Panay (0.0046, 0.0040; Table 1) this was similar to what was detected in host diversity measures at these sites.

Discussion

Host Genetic Architecture

298 The genetic structure of the *E. albatrossae* sampled for this study indicates that
299 geographical location impacts host distribution. Island effects, as reported by the
300 location and amount of variance during AMOVA analysis were detected in host genetic
301 data, further supporting that geologic origin, physical geography, and possibly
302 environmental factors have shaped the distribution of host squid in the region (among
303 Islands, d.f=3, SS= 1340.934, VC= 23.8035, PV= 66.61%; Table 3). The genetic fixation
304 observed in the host genetic data, reported as F_{ST}, indicate that genetic flow is limited
305 throughout the region and populations are genetically isolated from each other (F_{ST}=
306 0.70897, $p<0.0001$; Table 3). This is reflected in the three distinct host networks
307 detected in the Philippines (Fig. 2), and suggests geography may be influencing host
308 genetic exchange and distribution. This could be due to the benthic lifestyle adult
309 *Euprymna* squid (~5-8 cm) lead as adults rarely migrate, however the semi pelagic
310 nature of newly hatched squid (~3-10 mm) would allow water flow to transport juveniles
311 to novel locations (Kimbell et al., 2002; Villanueva, Vidal, Fernández-Álvarez, &
312 Nabhitabhata, 2016a). Two of the distinct networks occur in the central island chain, and
313 while having similar geological origin, show no genetic connection in habitats that are
314 homogeneous (Allen & Werner, 2002). Clade 4-7 is distinctly made up of mostly squid
315 haplotypes detected around the island of Panay, with small contributions from
316 populations around Negros (Haplotypes 21, 22, 23, 33, Fig. 2). This indicates that
317 populations may have been fragmented due to the result of geologic activity in the
318 region, seasonal changes in currents, or even modern day commercial fishing
319 management- which have all been shown to influence fragmentation of marine habitats
320 in the area (Abesamis, Russ, & Alcala, 2006; Huang, Wu, Zhao, Chen, & Wang, 1997;

Savina & White, 1986; Wyrtki, 1961; Zhou, Ru, & Chen, 1995). Habitat fragmentation was also inferred within clades 2-11 and 3-1 (Table 4), where genetic data indicates restricted gene flow with isolation by distance. Clade 2-11 (Fig. 2), comprised solely of host haplotypes detected around Panay, is closely connected (in some cases only one base pair difference between haplotypes; Fig. 2) to haplotypes with relatively small genetic contributions from the island of Negros. This indicates that the physical geography of the central island chain may be restricting host movement and genetic exchange. Additionally, Clade 3-7 (Fig. 2) inferred contiguous range expansion between populations sampled from the islands of Panay and Negros despite these populations being isolated geographically (Table 4), suggesting that the alternating direction of prevailing currents in the region is one mechanism of dispersal as well as isolation for these squids.

The divergence of the equatorial current (EC) as it approaches the Philippines from the east influences the directional flow of water through the central island chain, particularly north of the island of Panay and south of the islands of Negros and Cebu (Fig. 5; (Huang et al., 1997; Wyrtki, 1961). The amount and speed of water that is funneled around or through the central islands depends on the time of year, and is reflected in the patterns detected in host haplotype networks (Wyrtki, 1961). In late winter/early spring, water flows from east to west from the San Bernardino Strait, south of Masbate and finally around the north of Panay toward the Sulu Sea (Fig. 5A). This flow pattern coupled with the flow of the southern divergence of the EC allows for genetic exchange between geographically isolated populations from Panay, Cebu, and Negros (Fig. 2; Clade 4-1 and 4-7) by allowing squid to be transported to areas they

344 could not reach by themselves. As spring continues, the divergence pattern changes,
345 and only a moderate east to west flow of water north of Panay is produced, while the
346 bulk of the southern divergence is shifted south of the island of Mindanao, temporarily
347 isolating squid populations south of Negros and Cebu from populations to the north of
348 Panay (Fig. 5B). During the summer months (June-August), waters within the central
349 island chain are relatively still, with most of the equatorial current diverted northeast of
350 Luzon and southeast of Mindanao, circling around to the north, into the Sulu Sea (Fig.
351 5C). This isolates the central islands from the western island of Palawan, and further
352 prohibits exchange across the Sulu Sea. The two predominant haplotypes detected from
353 our squid data (Haplotypes 1 and 5; Fig. 2) are separated by only one base pair
354 difference even though the populations that contribute to these haplotypes are
355 separated by physical barriers. This further supports the notion that currents may be
356 influencing the prevalence and direction of gene flow between Panay squid populations
357 and other squid assemblages to the south.
358 While Clade 4-1 had no valid inference (Table 4), clade 3-1, which is fully nested
359 within 4-1 (Fig. 2), had an inference of restricted gene flow with isolation by distance.
360 This indicates that these habitats may have changed over time and influenced the
361 genetic flow between once connected populations of host squid. A third separate
362 network (Clade 4-2, Fig. 2) consisted solely of samples collected around the western
363 island of Palawan provides evidence that the unique geologic origin of Palawan may
364 have fragmented a once continuous population of host animals (Sathiamurthy & Voris,
365 2006; Wallace, 1863; Zhou et al., 1995). Likewise, factors such as the deep water
366 thermocline in the middle of the Sulu Sea, and changes in surface water transport (Fig.

5D) may have isolated these populations from others in the central islands (Chen, Yeh, Chen, & Huang, 2015; Huang et al., 1997; Miao, Thunell, & Anderson, 1994; Stuecker, Timmermann, Jin, McGregor, & Ren, 2013).

Symbiont genetic architecture

Symbiont genetic data indicate that *Vibrio* bacteria seem to be able to mitigate the barriers that restrict host genetic exchange. Analysis (AMOVA) of total symbiont genetic data reveal that most of the genetic variation observed lies within each population in contrast to the partitioning of variation from host genetic data (d.f.= 186, SS= 438.538, VC= 2.6704, PV= 80.08%; Table 3). The level of genetic diversity of the total symbiont population in the region is also indicative of symbiont gene flow between populations of hosts that are isolated from one another (F_{ST}= 0.1991, Table 3). One contiguous haplotype network was detected in the symbiont genetic data (Figs. 3, 4) revealing several genetic connections between symbionts collected from host squid that are genetically and geographically isolated from one another (Fig. 2). The predominant haplotypes found within symbiont genetic data (Haplotypes 1, 3, 8, 57, and 58; Fig. 3) are comprised of samples from all the islands, regardless of geological origin or physical position. In contrast to the pattern detected in host data, symbiont genetic data displays connections between the western island of Palawan and the central Islands to the east (Haplotypes 1, 3, 8, 57, and 58; Fig. 3). Interestingly, *Vibrio* haplotype 8 (Fig. 3) consisting of samples primarily from Palawan, has only 6 base pair changes from the more diverse haplotype 1 (Fig. 3). The composition of these two haplotypes (8 and 1, Fig. 3) is quite different and could be a result of multiple introgressions of these

390 populations by neighboring and distant *V. fischeri* symbionts displaced from their native
391 range. When including intermediate haplotypes from Cebu (Haplotype 40, Fig. 3) and
392 Negros (Haplotype 16, 11, and 12; Fig. 3), there is evidence for connection between
393 symbiont populations where hosts are restricted. While geography appears to have little
394 influence on the population structure of symbiotic *V. fischeri* in this region,
395 oceanographic currents may be influencing the ability of bacteria to cross barriers that
396 restrict hosts.
397
398 **Geography, geologic history, and environmental conditions**
399 Results from this study indicate that geography plays a role in host squid
400 distribution, without demonstrating a significant influence on symbiont distribution. The
401 disparity in these patterns may be a result of differences in dispersal methodology
402 between mutualist partners, *i.e.* host squid have a limited range as adults and rarely
403 travel far from their birthplace due to the limited dispersal ability of direct developing,
404 benthic hatchlings (Kimbell et al., 2002; Villanueva, Vidal, Fernández-Álvarez, &
405 Nabhitabhata, 2016b). Conversely, symbiotically viable *Vibrio* bacteria are cycled out of
406 the host daily exposing them to environmental factors (i.e. currents) that allow for
407 movement into novel areas where they are able to recruit into a new host. While bacteria
408 alone cannot cross great expanses of ocean, the use of rafting has been shown to be an
409 effective dispersal mechanism for marine bacteria like *V. fischeri* (Jones et al., 2006;
410 Theil & Gutow, 2005). The ability for vibrios to cross great expanses of oceans has been
411 previously reported in other marine bacteria and undoubtedly will allow symbiotically

412 viable vibrios to be shuttled to new areas and novel hosts (González-Escalona, Gavilan,
413 Brown, & Martinez-Urtaza, 2015).

414 Prevailing currents in and around the central Philippine island chain vary in
415 direction and magnitude seasonally (Wyrtki, 1961). Since *E. albatrossae* breed all year
416 long, this change in directionality may provide newly hatched squid the opportunity to be
417 carried to new areas, despite their otherwise limited dispersal ability, while being cut off
418 from other available habitats when the prevailing currents change (Hanlon, Claes,
419 Ashcraft, & Dunlap, 1997). The pattern of direction in the symbiont genetic data
420 presented here indicates that introgression across the Sulu Sea, which appears to be a
421 biogeographical margin for host animals, is facilitated by the directional flow of water
422 during the monsoon season (Huang et al., 1997). *Euprymna* hatchlings are known to be
423 “pelagic”, that is, they linger in the water column before settling to their benthic lifestyle
424 (Moltschaniwskyj & Doherty, 1995). This also might heighten the ability of host
425 populations to move to new localities.

426 Geological changes and the physical oceanography of this region may also
427 explain the patterns detected in the genetic data. Glacial maximum sea levels exposed
428 portions of what was host native range within the central island chain, creating a
429 disconnect between populations in the west and central island squid assemblages
430 (Gaither & Rocha, 2013; Gordon, 2005; Zhou et al., 1995). During glacial norms, hosts
431 are restricted by a deep-water thermocline that has persisted since before the Holocene,
432 between Palawan and the central island chain (Miao et al., 1994). Fluctuating sea level
433 during glacial cycles as well as Cenozoic volcanic uprising of the central Visayas may
434 also explain the disjunctive distribution of host animals across this region (Miao et al.,

435 1994; Zhou et al., 1995). Given that many of the more abundant haplotypes examined
436 have prevalence in localities that are geographically distinct, this provides additional
437 evidence that host populations have been established well beyond the geological history
438 of the Philippines (e.g., Palawan).

439 While previous research has shown that symbiont gene flow can be restricted by
440 temperature, symbionts in this region seem to be able to mitigate environmental barriers
441 which hosts cannot, crossing geographical and biological barriers with apparent ease
442 (Nishiguchi, 2000). Symbiont gene flow demonstrates a current dependent directionality
443 of introgression by vibrios from the central islands west to Palawan in the winter, and
444 west to east in the summer months (Huang et al., 1997). The El Niño Southern
445 Oscillation has also been shown to influence not only sea surface temperatures, wind
446 direction, and rainfall in this region, but also the position of this deep water thermocline ,
447 further isolating local populations of host squid while not restricting symbiont distribution
448 (Chen et al., 2015; Stuecker et al., 2013). While other Indo-west Pacific and
449 Mediterranean populations of *Vibrio* demonstrate that some degree of host specificity,
450 geography, or other environmental factors can impact symbiont genetic architecture ,
451 findings from this study indicate that geography alone cannot explain symbiont
452 distribution, and that physical factors (e.g., currents) are important drivers of microbial
453 diversity in the region (Jones et al., 2006; Zamborsky & Nishiguchi, 2011).

454 Beneficial associations like the sepiolid squid-*Vibrio* mutualism will undoubtedly
455 be impacted by reduction of available habitat, highlighting the importance of
456 investigating the influence geography has on symbiont prevalence and distribution.
457 Findings from this study point to a need to better understand the mechanisms that will

458 impact symbiotic associations across a changing landscape, and what factors will
459 influence the fitness of beneficial microbes when they are moved to a novel habitat. Our
460 findings have provided clues as to how established populations of host squids are the
461 foundation for symbiont population structure, yet abiotic factors still influence where
462 vibrios can move and establish new populations.
463
464 **ACKNOWLEDGEMENTS**
465 The authors would like to thank all the collaborators, lab technicians, fisherman, and
466 local merchants without whom this research would not be possible. Without their help,
467 guidance, generosity, hospitality and invaluable local knowledge this project would not
468 be complete. We would also like to thank the administrators, staff, and technicians at the
469 Southeast Asian Fisheries Development Center (SEAFDEC), Siliman University
470 (Negros), the University of San Carlos (Cebu), and Western Philippine University
471 (Palawan) for providing us with lab facilities, transport, and local contacts.
472 We would like to thank M. Sedano and N. Nourabadi for help with collections during the
473 2012 and 2013 field seasons. Funding for this project was supported by New Mexico
474 State University Honors College International fellowship (K.E.T.), Howard Hughes
475 Medical Institute 52008103 (K.E.T.), Department of Biology at NMSU, National Institute
476 of Health Rise to the Postdoctorate-R25GM061222 (R.L.C.), American Association for
477 the Advancement of Science-WIRC/National Science Foundation (M.K.N.), University of
478 New Mexico-ASERT (IRACDA-NIH K12GM088021 (M.K.N.), American Society for
479 Microbiology International Professorship in Asia (M.K.N.), and the NMSU Foundation
480 (M.K.N.).

Data Accessibility:

- Final DNA sequences: Genbank accessions : *Euprymna albatrossae* (COI;MF379363 - MF379405) ; *Vibrio fischeri* (*gapA*; MF379406 - MF379465)

Author Contributions:

1. R.L. Coryell- Extracted DNA, amplified genes of interest, aligned and analyzed sequence data, wrote paper.
2. K.E. Turnham- Collected samples, generated data, analyzed data, co-wrote paper.
3. E.G.J. Ayson- Coordinated collecting of sample animals as well as provided laboratory facilities for processing of animals and culturing of symbiotic bacteria.
4. A. Alacala- Coordinated collecting of sample animals as well as provided laboratory facilities for processing of animals and culturing of symbiotic bacteria.
5. F. Soto- Coordinated collecting of sample animals as well as provided laboratory facilities for processing of animals and culturing of symbiotic bacteria.
6. B. Gonzales- Coordinated collecting of sample animals as well as provided laboratory facilities for processing of animals and culturing of symbiotic bacteria.
7. M.K. Nishiguchi- Coordinated and obtained project funding, initial experimental design, collection and processing of sample animals, co-author of paper, contributing editing and review of manuscript.

REFERENCES

Abesamis, R. A., Russ, G. R., & Alcala, A. C. (2006). Gradients of abundance of fish across no-take marine reserve boundaries: evidence from Philippine coral reefs. *Aquatic Conservation: Marine and Freshwater Ecosystems, 16*(4), 349-371. doi:10.1002/aqc.730

Alcazar, D. S., & Kochzius, M. (2016). Genetic population structure of the blue sea star *Linckia laevigata* in the Visayas (Philippines). *Journal of the Marine Biological Association of the United Kingdom, 96*(3), 707-713. doi:10.1017/S0025315415000971

Allen, G. R., & Werner, T. B. (2002). Coral reef fish assessment in the 'coral triangle'of southeastern Asia. *Environmental Biology of Fishes, 65*(2), 209-214. doi:10.1023/A:1020093012502

Boettcher, K., Ruby, E., & McFall-Ngai, M. (1996). Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *Journal of Comparative Physiology A, 179*(1), 65-73. doi: doi:10.1007/BF00193435

Bongrand, C., Koch, E. J., Moriano-Gutierrez, S., Cordero, O. X., McFall-Ngai, M., Polz, M. F., & Ruby, E. G. (2016). A genomic comparison of 13 symbiotic Vibrio fischeri isolates from the perspective of their host source and colonization behavior. *The ISME Journal, 10*(12), 2907-2917. doi:10.1038/ismej.2016.69

Calderon, I., Garrabou, J., & Aurelle, D. (2006). Evaluation of the utility of COI and ITS markers as tools for population genetic studies of temperate gorgonians. *Journal of Experimental Marine Biology and Ecology, 336*, 184-197. doi:10.1016/j.jembe.2006.05.006

Carpenter, K. E., & Springer, V. G. (2005). The center of the center of marine shore fish biodiversity: the Philippine Islands. *Environmental Biology of Fishes, 72*(4), 467-480. doi:10.1007/s10641-004-3154-4

Chen, C.-T., Yeh, Y.-T., Chen, Y.-C., & Huang, T.-H. (2015). Seasonal and ENSO-related interannual variability of subsurface fronts separating West Philippine Sea waters from South China Sea waters near the Luzon Strait. *Deep Sea Research Part I: Oceanographic Research Papers, 103*, 13-23. doi:10.1016/j.dsr.2015.05.002

Esselstyn, J. A., Oliveros, C. H., Moyle, R. G., Peterson, T. A., McGuire, J. A., & Brown, R. M. (2010). Integrating phylogenetic and taxonomic evidence illuminates complex biogeographic patterns along Huxley's modification of Wallace's Line. *Journal of Biogeography, 37*(11), 2054-2066. doi:10.1111/j.1365-2699.2010.02378.x

Excoffier, L., & Lischer, H. E. L. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources, 10*(3), 564-567. doi:10.1111/j.1755-0998.2010.02847.x

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*(5), 294-299.

Gaither, M. R., & Rocha, L. A. (2013). Origins of species richness in the Indo-Malay-Philippine biodiversity hotspot: evidence for the centre of overlap hypothesis. *Journal of Biogeography, 40*(9), 1638-1648. doi:10.1111/jbi.12126

Gaither, M. R., Toonen, R. J., Robertson, R. D., Planes, S., & Bowen, B. W. (2010). Genetic evaluation of marine biogeographical barriers: perspectives from two widespread Indo-Pacific snappers (*Lutjanus kasmira* and *Lutjanus fulvus*). *Journal of Biogeography, 37*(1), 133-147. doi:10.1111/j.1365-2699.2009.02188.x

González-Escalona, N., Gavilan, R. G., Brown, E. W., & Martinez-Urtaza, J. (2015). Transoceanic Spreading of Pathogenic Strains of Vibrio parahaemolyticus with Distinctive Genetic Signatures in the recA Gene. *PLOS ONE, 10*(2). doi:10.1371/journal.pone.0117485

Gordon, A. L. (2005). Oceanography of the Indonesian Seas. *Oceanography, 18*(4), 14-27. doi:10.5670/oceanog.2005.18

Hanlon, R. T., Claes, M. F., Ashcraft, S. E., & Dunlap, P. V. (1997). Laboratory culture of the sepiolid squid Euprymna scolopes: a model system for bacteria-animal symbiosis. *The Biological Bulletin*.

Hellberg, M. E., Burton, R. S., Neigel, J. E., & Palumbi, S. R. (2002). Genetic assessment of connectivity among marine populations. *Bulletin of marine ….*

Herring, P. J. (1977). Luminescence in cephalopods and fish. *Symposia of the Zoological Society of London, 38*, 127-159.

Horne, J. B. (2014). Thinking outside the barrier: neutral and adaptive divergence in Indo-Pacific coral reef faunas. *Evolutionary Ecology, 28*(6), 991-1002.

Huang, C. Y., Wu, S. F., Zhao, M., Chen, M. T., & Wang, C. H. (1997). Surface ocean and monsoon climate variability in the South China Sea since the last glaciation. *Marine Micropaleontology, 32*(1-2), 71-94 doi:https://doi.org/10.1016/S0377-8398(97)00014-5

Huxley, T. H. (1868). *On the classification and distribution of the Alectoromorphae and Heteromorphae*. London: Royal Institution.

Jacobs, H. T., & Grimes, B. (1986). Complete nucleotide sequences of the nuclear pseudogenes for cytochrome oxidase subunit I and the large mitochondrial ribosomal RNA in the sea urchin Strongylocentrotus purpuratus. *Journal of Molecular Biology, 187*(4), 509 - 527. doi:https://doi.org/10.1016/0022-2836(86)90330-X

Jones, B. W., Lopez, J. E., Huttenburg, J., & Nishiguchi, M. K. (2006). Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Molecular Ecology, 15*(14), 4317-4329. doi:10.1111/j.1365-294X.2006.03073.x

Jones, B. W., & Nishiguchi, M. K. (2004). Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). *Marine Biology, 144*(6), 1151-1155. doi:10.1007/s00227-003-1285-3

Juinio-Menez, M., Magsino, R., Ravago-Gotanco, R., & Yu, E. (2003). Genetic structure of *Linckia laevigata* and *Tridacna crocea* populations in the Palawan shelf and shoal reefs. *Marine Biology, 142*(4), 717-726.

593 Kimbell, J. R., McFall-Ngai, M. J., & Roderick, G. K. (2002). Two genetically distinct
594 populations of bobtail squid, *Euprymna scolopes*, exist on the island of O'ahu.
595 *Pacific science*. doi:10.1353/psc.2002.0024
596 Kool, J. T., Paris, C. B., Barber, P. H., & Cowen, R. K. (2011). Connectivity and the
597 development of population genetic structure in Indo-West Pacific coral reef
598 communities. *Global Ecology and Biogeography, 20*(5), 695-706.
599 doi:10.1111/j.1466-8238.2010.00637.x
600 Lemer, S., Combosch, D., Sotto, F., Giribet, G., Dumale, D., & Soliman, V. (2016). The
601 family Pinnidae (Bivalvia) in the Philippine archipelago: observations on its
602 distribution and phylogeography. *NAUTILUS*.
603 Lessios, H. A., Kane, J., & Evolution, R.-D. R. (2003). Phylogeography of the pantropical
604 sea urchin Tripneustes: contrasting patterns of population structure between
605 oceans. *Evolution*. doi:10.1554/02-681
606 Liu, S.-Y. V., Chang, F.-T., Borsa, P., Chen, W.-J., & Dai, C.-F. (2014). Phylogeography
607 of the humbug damselfish, *Dascyllus aruanus* (Linnaeus, 1758): evidence of Indo-
608 Pacific vicariance and genetic differentiation of peripheral populations. *Biological*
609 *Journal of the Linnean Society, 113*(4), 931-942. doi:10.1111/bij.12378
610 Lohman, D. J., de Bruyn, M., Page, T., von Rintelen, K., Hall, R., Ng, P., . . . von
611 Rintelen, T. (2011). Biogeography of the Indo-Australian Archipelago. *Annual*
612 *Review of Ecology, Evolution, and Systematics, 42*(1), 205-226.
613 doi:10.1146/annurev-ecolsys-102710-145001
614 Magsino, R. M., Ravago, R. G., & Juinio-Menez, M. A. (2002). *Genetic relationship of*
615 *Linckia laevigata color morphs in the Kalayaan Islands Group, western*
616 *Philippines: preliminary evidence*. Paper presented at the Proceedings of the 9th
617 International Coral Reef Symposium, Bali, Indonesia.
618 http://www.coremap.or.id/downloads/ICRS9th-Magsino.pdf
619 McFall-Ngai, M. (2014). Divining the Essence of Symbiosis: Insights from the Squid-
620 Vibrio Model. *PLoS Biology, 12*(2). doi:10.1371/journal.pbio.1001783
621 McFall-Ngai, M., Heath-Heckman, E., Gillette, A. A., Peyer, S. M., & Harvie, E. A.
622 (2012). The secret languages of coevolved symbioses: Insights from the
623 *Euprymna scolopes–Vibrio fischeri* symbiosis. *Seminars in Immunology, 24*(1), 3-
624 8. doi:10.1016/j.smim.2011.11.006
625 Miao, Q., Thunell, R. C., & Anderson, D. M. (1994). Glacial-Holocene carbonate
626 dissolution and sea surface temperatures in the south China and Sulu seas.
627 *Paleoceanography, 9*(2), 269-290. doi:10.1029/93PA02830
628 Moltschaniwskyj, N. A., & Doherty, P. J. (1995). Cross-shelf distribution patterns of
629 tropical juvenile cephalopods sampled with light-traps. *Marine and Freshwater*
630 *Research, 46*(4), 707-714. doi:10.1071/MF9950707
631 Nelson, K., Whittam, T. S., & Selander, R. K. (1991). Nucleotide polymorphism and
632 evolution in the glyceraldehyde-3-phosphate dehydrogenase gene (gapA) in
633 natural populations of Salmonella and Escherichia coli. *Proceedings of the*
634 *National Academy of Sciences, 88*(15), 6667-6671. doi:10.1073/pnas.88.15.6667
635 Nishiguchi, M. K. (2000). Temperature affects species distribution in symbiotic
636 populations of Vibrio spp. *Applied and environmental microbiology, 66*(8), 3550-
637 3555. doi:http://dx.doi.org/10.1128/AEM.66.8.3550-3555.2000

Nishiguchi, M. K., & Nair, V. S. (2003). Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. *International Journal of Systematic and Evolutionary Microbiology, 53*(6), 2019-2026. doi:10.1099/ijs.0.02792-0

Nyholm, S. V. (2004). The winnowing: establishing the squid–vibrio symbiosis. *Nature reviews. Microbiology, 2*(8), 632-642. doi:10.1038/nrmicro957

Nyholm, S. V., & Nishiguchi, M. K. (2008). The evolutionary ecology of a sepiolid squid-vibrio association: from cell to environment. *Vie et milieu, 58*(2), 175.

Nyholm, S. V., Stabb, E. V., Ruby, E. G., & McFall-Ngai, M. J. (2000). Establishment of an animal–bacterial association: Recruiting symbiotic vibrios from the environment. *Proceedings of the National Academy of Sciences, 97*(18), 10231-10235. doi:10.1073/pnas.97.18.10231

Palumbi, S., Grabowsky, G., Duda, T., Geyer, L., & Tachino, N. (1997). Speciation and Population Genetic Structure in Tropical Pacific Sea Urchins. *Evolution, 51*(5), 1506. doi:10.2307/2411203

Palumbi, S. R. (1994). Genetic divergence, reproductive isolation, and marine speciation. *Annual review of ecology and systematics*. doi:10.1146/annurev.es.25.110194.002555

Posada, D., Crandall, K. A., & Templeton, A. R. (2000). GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology, 9*(4), 487-488. doi:10.1046/j.1365-294x.2000.00887.x

Ravago-Gotanco, R. G., Magsino, R. M., & Juinio-Menez, M. A. (2007). Influence of the North Equatorial Current on the population genetic structure of *Tridacna crocea* (Mollusca: Tridacnidae) along the eastern Philippine seaboard. *Marine Ecology Progress Series, 336*, 161-168.

Roberts, C. M., McClean, C. J., Veron, J. E. N., Hawkins, J. P., Allen, G. R., McAllister, D. E., . . . Werner, T. B. (2002). Marine biodiversity hotspots and conservation priorities for tropical reefs. *Science, 295*(5558), 1280-1284. doi:10.1126/science.1067728

Sathiamurthy, E., & Voris, H. K. (2006). Maps of Holocene sea level transgression and submerged lakes on the Sunda Shelf. *The Natural History Journal of ….*

Savina, G. C., & White, A. T. (1986). A tale of two islands: some lessons for marine resource management. *Environmental Conservation, 13*(2), 107-113. doi:10.1017/S0376892900036699

Siler, C. D., Oaks, J. R., Esselstyn, J. A., Diesmos, A. C., & Brown, R. M. (2010). Phylogeny and biogeography of Philippine bent-toed geckos (Gekkonidae: Cyrtodactylus) contradict a prevailing model of Pleistocene diversification. *Molecular phylogenetics and evolution, 55*(2), 699-710. doi:10.1016/j.ympev.2010.01.027

Sorenson, L., Allen, G. R., Erdmann, M. V., Dai, C.-F., & Liu, S.-Y. (2014). Pleistocene diversification of the *Pomacentrus coelestis* species complex (Pisces: Pomacentridae): historical biogeography and species boundaries. *Marine Biology, 161*(11), 2495-2507. doi:10.1007/s00227-014-2521-8

Soto, W., Gutierrez, J., Remmenga, M., & Nishiguchi, M. (2009). Salinity and Temperature Effects on Physiological Responses of Vibrio fischeri from Diverse Ecological Niches. *Microbial Ecology*. doi:10.1007/s00248-008-9412-9

Stuecker, M. F., Timmermann, A., Jin, F.-F., McGregor, S., & Ren, H.-L. (2013). A combination mode of the annual cycle and the El Nino/Southern Oscillation. *Nature Geoscience, 6*(7), 540-544. doi:10.1038/ngeo1826

Templeton, A. R., & Sing, C. F. (1993). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics, 134*(2), 659-669.

Theil, M., & Gutow, L. (2005). The ecology of rafting in the marine environment. II. The rafting organisms and community. *Oceanography and Marine Biology: An Annual Review, 43*, 279-418.

Thompson, F. L., Gomez-Gil, B., Vasconcelos, A., & Sawabe, T. (2007). Multilocus Sequence Analysis Reveals that Vibrio harveyi and V. campbellii Are Distinct Species. *Applied and Environmental Microbiology, 73*(13), 4279-4285. doi:10.1128/AEM.00020-07

Tonon, L. A., de Silva, B. O., Moreira, A. B., Valle, C., Alves, N., Cavalcanti, G., . . . Thompson, F. L. (2015). Diversity and ecological structure of vibrios in benthic and pelagic habitats along a latitudinal gradient in the Southwest Atlantic Ocean. *PeerJ, 3*. doi:10.7717/peerj.741

Villanueva, R., Vidal, E. A. G., Fernández-Álvarez, F., & Nabhitabhata, J. (2016a). Early mode of life and hatchling size in Cephalopod Molluscs: Influence on the species distributional ranges. *PLOS ONE, 11*(11), e0165334. doi:10.1371/journal.pone.0165334

Villanueva, R., Vidal, E. A. G., Fernández-Álvarez, F., & Nabhitabhata, J. (2016b). Early mode of life and hatchling size in Cephalopod Molluscs: Influence on the species distributional ranges. *PLOS ONE, 11*(11), e0165334. doi:10.1371/journal.pone.0165334

Wallace, A. R. (1860). On the Zoological Geography of the Malay Archipelago. *Journal of the Proceedings of the Linnean Society of London. Zoology, 4*(16), 172-184. doi:10.1111/j.1096-3642.1860.tb00090.x

Wallace, A. R. (1863). On the physical geography of the Malay Archipelago. *The Journal of the Royal Geographical Society of London, 7*(5), 206-213. doi:1798448

Wollenberg, M. S., & Ruby, E. G. (2009). Population structure of Vibrio fischeri within the light organs of Euprymna scolopes squid from Two Oahu (Hawaii) populations. *Applied and environmental microbiology, 75*(1), 193-202. doi:10.1128/aem.01792-08

Wyrtki, K. (1961). Physical oceanography of the Southeast Asian waters. *Scripps Institution of Oceanography*.

Zamborsky, D. J., & Nishiguchi, M. K. (2011). Phylogeographical patterns among Mediterranean sepiolid squids and their Vibrio symbionts: environment drives specificity among sympatric species. *Applied and environmental microbiology, 77*(2), 642-649. doi:10.1128/AEM.02105-10

Zhou, D., Ru, K., & Chen, H. (1995). Kinematics of Cenozoic extension on the South China Sea continental margin and its implications for the tectonic evolution of the region. *Tectonophysics, 251*, 161-177.

Figure Legends

Figure 1. Sampling location where host squid were collected during the months of May, June, July, and August of 2010-2013 and 2015.

Figure 2. TCS nested haplotype network generated from *E. albatrossae* molecular data acquired from animals captured in the Philippines during the years 2010-2015. Each line in the diagram represents one base pair mutational step between haplotypes. Black circles represent unsampled mutational steps connecting haplotypes. The size of each circle is indicative of the number of sequences that make up that haplotype, with the color of each circle representing the geographic origin of the sequence data and its proportion of the total haplotype. Each haplotype is represented by a two digit indicator with the dotted line enclosures indicating the nesting hierarchy. Each nesting level is labeled with a dashed two-digit label.

Figure 3. TCS haplotype network generated from *Vibrio fischeri* molecular data acquired from isolates harvested from squid light organs in the Philippines during the years 2010-2015. Each line in the diagram represents one base pair mutational step between haplotypes. Black circles represent un-sampled haplotypes. The size of each circle is indicative of the number of sequences that make up that haplotype, with the color of each circle representing the geographic origin of the sequence data and its proportion of the total haplotype. Each haplotype is represented by a two-digit indicator.

Figure 4. Nested *Vibrio fischeri* haplotype network generated from molecular data acquired in the Philippines during 2010, and 2012 through 2015. Each haplotype is represented by a two-digit identifier (see Fig. 3), with each hierarchical nesting level represented by a 2 to 3 digit dashed identifier and enclosed within dashed and dotted lines. Lines between haplotypes represent the mutational steps required to transition from one genetic station to another, with the small black dots representing un-sampled haplotypes.

Figure 5. Maps of surface currents in the Philippine islands during: A) Winter and early Spring (Dec-Feb), B) Late Spring (Mar-May) C) Early Summer (June-July), D) Late Summer/ Fall (Aug-Nov). Adapted from Wyrtki 1961.

Table 1. Geographic location, sampling years, and sites where *Euprymna albatrossae* and *Vibrio fischeri* were collected in the Philippines during the months of May, June, July, and August of the years listed below. All squids were wild caught adults and approximately 5 cm in mantle length.

Site Name	Abbreviation	Years Collected	Host n	Coordinates (Decimal Degrees)	Host □	*Vibrio* □
Cordova, Mactan	CVCP	2015	9	124.004 10.317	.00135	0.0051
Magellan Bay, Mactan	MGCP	2015	10	124.009 10.528	0.0138	0.0046
Dumaguete, Negros	DBNP	2013	2	123.269 9.186	0.1444	0.0074
Sibulan, Negros	SIBNP	2013	8	123.276 9.396	0.0248	0.0061
Siliman, Negros	SBNP	2013	5	123.310 9.31	0.0885	0.0050
Puerto Bay, Palawan	PBPP	2013, 2015	9	118.733 9.733	0.0431	0.0048
Atabayan, Panay (SEAFDEC)	SEDEC	2010,2012,2013	8	122.400 10.667	0.0055	0.0085
Banate, Panay	BAN	2012	10	122.815 10.983	0.0067	0.0040
San Juan Barotac, Panay	SJBV	2012	7	122.872, 10.027	0.0467	0.0058
Santo Nino Sur, Panay	SNSP	2015	7	122.504 10.679	0.0441	0.0052
Parara, Panay	PARA	2013	6	122.353 10.700	0.0722	0.0076

Table 2. Primer names, sequence, and sequence source used in the amplification of the glyceraldehyde phosphate dehydrogenase (*gapA*) locus from *Vibrio fischeri*, and the cytochrome *c* oxidase subunit 1 (COI) locus from *Euprymna albatrossae* collected in the Philippines from 2010-2015.

Primer Name	Fragment Size	Primer Sequence	Source
gapAF	889 bp	5'-GGATTTGGCCGCATCGGCCG-3'	Jones *et al.* 2006; Zamborski and Nishiguchi 2011
gapAR		5'-GGATTTGGCCGCATCGGCCG-3'	
COI F	658 bp	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Jones *et al.* 2006; Nishiguchi *et al.* 2004
COI R		5'-GGTCAACAAATCATAAAGATATTGG-3'	

Table 3. Results of identical AMOVA analyses performed on host and symbiont genetic data.

Source of *E. albatrossae* Variation	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among Islands	3	1340.934	23.8035 *	66.61
Within Islands	7	143.175	1.4918	4.29
Within Populations	70	709.212	10.1316 *	29.10
Total	80	2193.321	34.8127	
Overall (F_{ST})		0.70897*		
Source of *V. fischeri* Variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among Islands	3	57.057	0.1799	5.52
Within Islands	7	66.639	0.4693*	14.40
Within Populations	168	438.538	2.6704*	80.08
Total	178	562.235	3.2596	
Overall (F_{ST})		0.1991*		

*, P< 0.001

Table 4. *Euprymna* clades that demonstrated significance during either a permutation contingency analysis or geographic distance analysis. Location of significance is indicated by (D_n), nested clade distance and/or (D_c) the within clade distance. I-T indicates the average distance between a tip clade and an interior clade. S or L indicates the distance measure is significantly small or large at the 5% inference level. Inference steps were performed using the automated inference key in GEODIS, part of the AneCA v1.2 population genetics analysis software platform (Posada, Crandall, & Templeton, 2000).

Clade	Nested Clade	Dist.	Value (S or L)	*P*	Inference Key Steps	Inference
.2-3	1-6(T)	D_n	102.87 S	0.0485	1,19,NO	Allopatric Fragmentation
	1-5(I)	D_c	23.288 S	0.0485		
		D_n	38.528 S	0.0485		
	I-T	D_n	-64.3381 S	0.0485		
2-11	1-21 (T)	D_c	0.0 S	0.0322	1,2,3,4,NO	Restricted gene flow with isolation by distance
3-1	2-1 (T)	D_c	54.307 S	0.0094	1,2,3,4,NO	Restricted gene flow with isolation by distance
3-7	2-9 (T)	D_n	97.411 L	0.048	1,2,11,12, NO	Contiguous range expansion
	2-11 (I)	D_c	31.471 S	0.0406		
		D_n	61.135 S	0.0417		
	I-T	D_c	-57.4872 S	0.0386		
		D_n	-36.2756 S	0.0428		
Total	4-1	D_c	75.7056 S	0.0002	1,2,IO	I-T Status undetermined: Inconclusive outcome
	4-2	D_c	0.000	>0.001		
		D_n	435.1782 L	>0.001		

4-7	D_c	67.0744 S	>0.001
	D_n	82.2657 S	

Table 5. *Vibrio fischeri* clades that demonstrated significance during either a permutation contingency analysis or geographic distance analysis. Location of significance is indicated by (D_n), nested clade distance and/or (D_c) the within clade distance. I-T indicates the average distance between a tip clade and an interior clade. S or L indicates the distance measure is significantly small or large at the 5% inference level. Inference steps were performed using the automated inference key in GEODIS, part of the AneCA v1.2 population genetics analysis software platform(Posada, Crandall, & Templeton, 2000).^{NS}= not significant.

Clade	Nested Clade	Dist.	Value (S or L)	*P*	Inference Key Steps	Inference
2-1	1-1 (T)	D_c	76.77 S	0.0218	1,19,20,2,3,4,NO	Restricted gene flow with isolation by distance (restricted dispersal by distance in non-sexual species)
		D_n	84.88 S	0.0408		
	1-27 (I)	D_n	117.3675	0.0043		
	I-T	D_c	-76.7722	0.0667^{NS}		
		D_n	32.4858	0.0043		
2-8	1-11(T)	D_c	196.5289 S	0.0183	1,19,20,2,3,4,NO	Restricted gene flow with isolation by distance (restricted dispersal by distance in non-sexual species)
3-5	2-11 (T)	D_n	99.7825 L	0.0139	1,2,3,4,NO	Contiguous range expansion
	2-12 (I)	D_c	0.833 S	0.0054		
		D_n	73.3769 S	0.0139		
	I-T	D_c	-71.6474 S	0.0054		
		D_n	-26.4056	0.0139		

4-2	3-2 (T)	D_c	240.9458 L	0.0197	1,2,11,12, NO	Contiguous range expansion
		D_n	241.0454 L	0.0045		
	3-3 (I)	D_c	104.4759 S	>0.001		
		D_n	176.6458 S	0.0042		
	I-T	D_c	-136.4699 S	>0.001		
		D_n	-64.3996 S	0.0041		
Total	4-1 (T)	D_c	104.559 S	>0.001	1,2,3,4,NO	Restricted gene flow with isolation by distance (restricted dispersal by distance in non-sexual species)
		D_n	111.3004 S	>0.001		
	4-3 (T)	D_c	99.2733	0.0508*		
		D_n	97.1363 S	0.0069		
	4-2 (I)	D_c	214.3525 L	>0.001		
		D_n	198.3175 L	>0.001		
	I-T	D_c	110.8023 L	>0.001		
		D_n	89.7202 L	>0.001		

Figure Legends

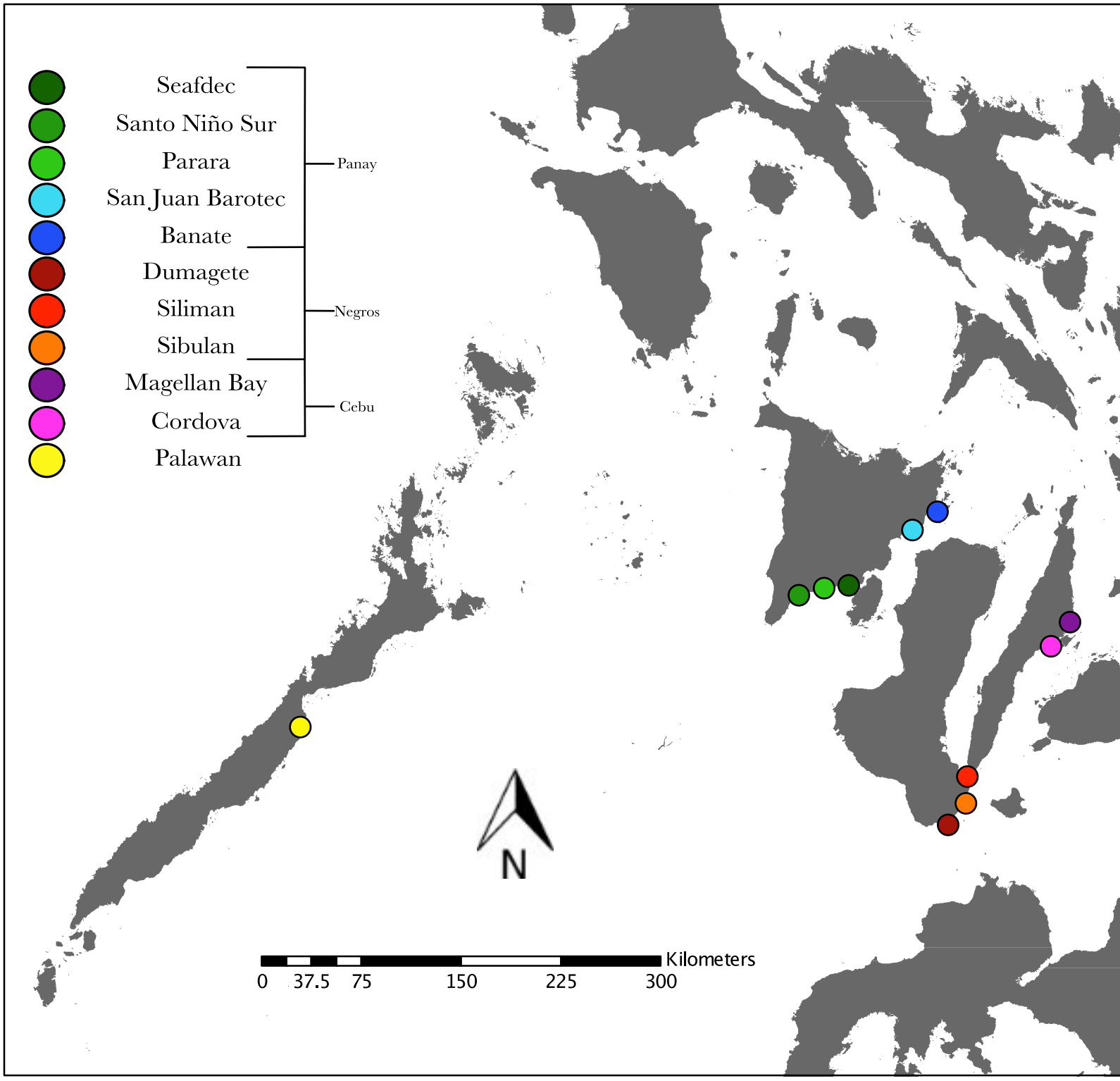
Figure 1. Sampling location where host squid were collected during the months of May, June, July, and August of 2010-2013 and 2015.

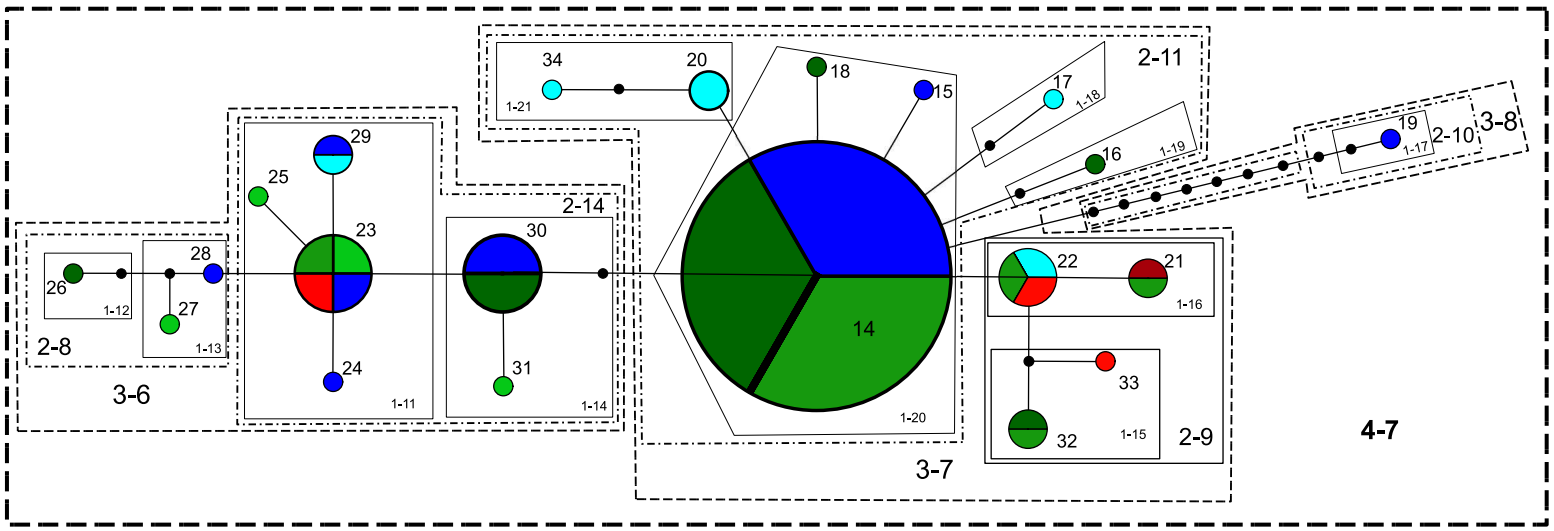
Figure 2. TCS nested haplotype network generated from *E. albatrossae* molecular data acquired from animals captured in the Philippines during the years 2010-2015. Each line in the diagram represents one base pair mutational step between haplotypes. Black circles represent unsampled mutational steps connecting haplotypes. The size of each circle is indicative of the number of sequences that make up that haplotype, with the color of each circle representing the geographic origin of the sequence data and its proportion of the total haplotype. Each haplotype is represented by a two digit indicator with the dotted line enclosures indicating the nesting hierarchy. Each nesting level is labeled with a dashed two-digit label.

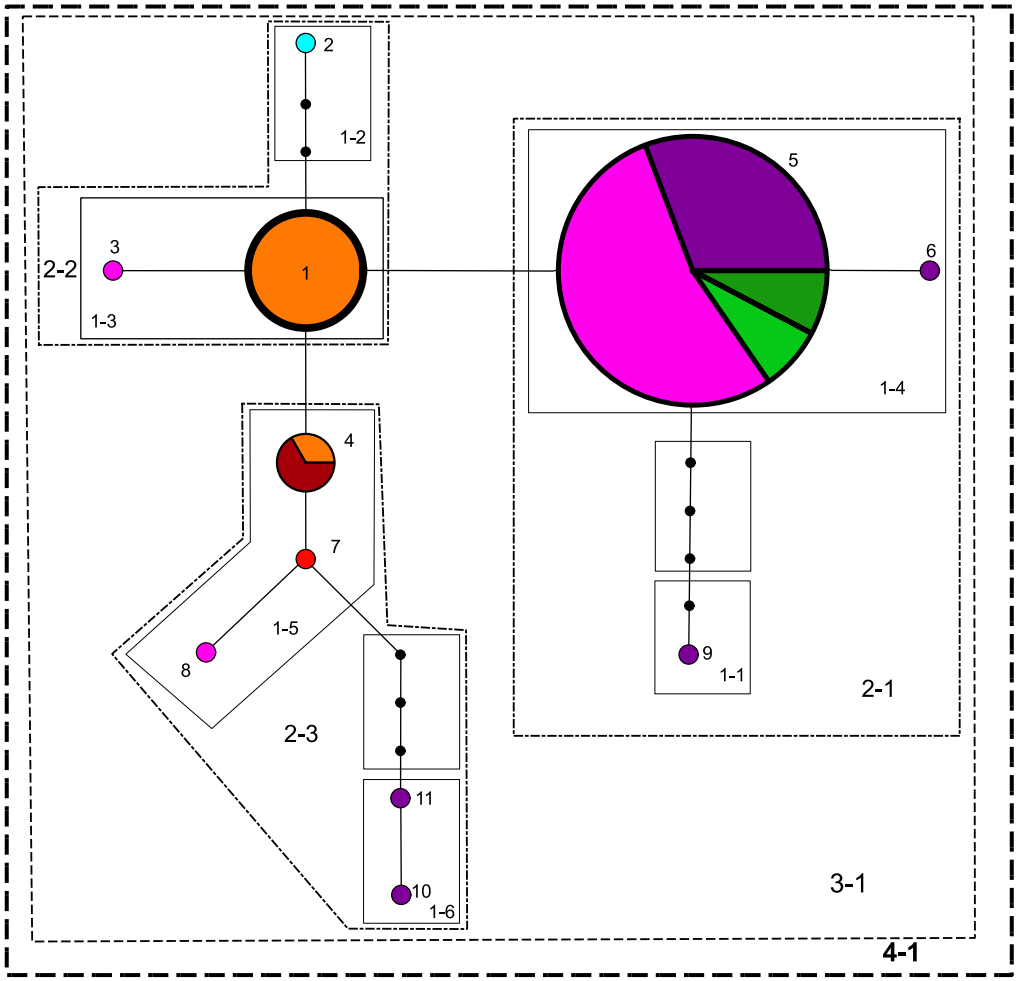
Figure 3. TCS haplotype network generated from *Vibrio fischeri* molecular data acquired from isolates harvested from squid light organs in the Philippines during the years 2010-2015. Each line in the diagram represents one base pair mutational step between haplotypes. Black circles represent un-sampled haplotypes. The size of each circle is indicative of the number of sequences that make up that haplotype, with the color of each circle representing the geographic origin of the sequence data and its proportion of the total haplotype. Each haplotype is represented by a two-digit indicator.

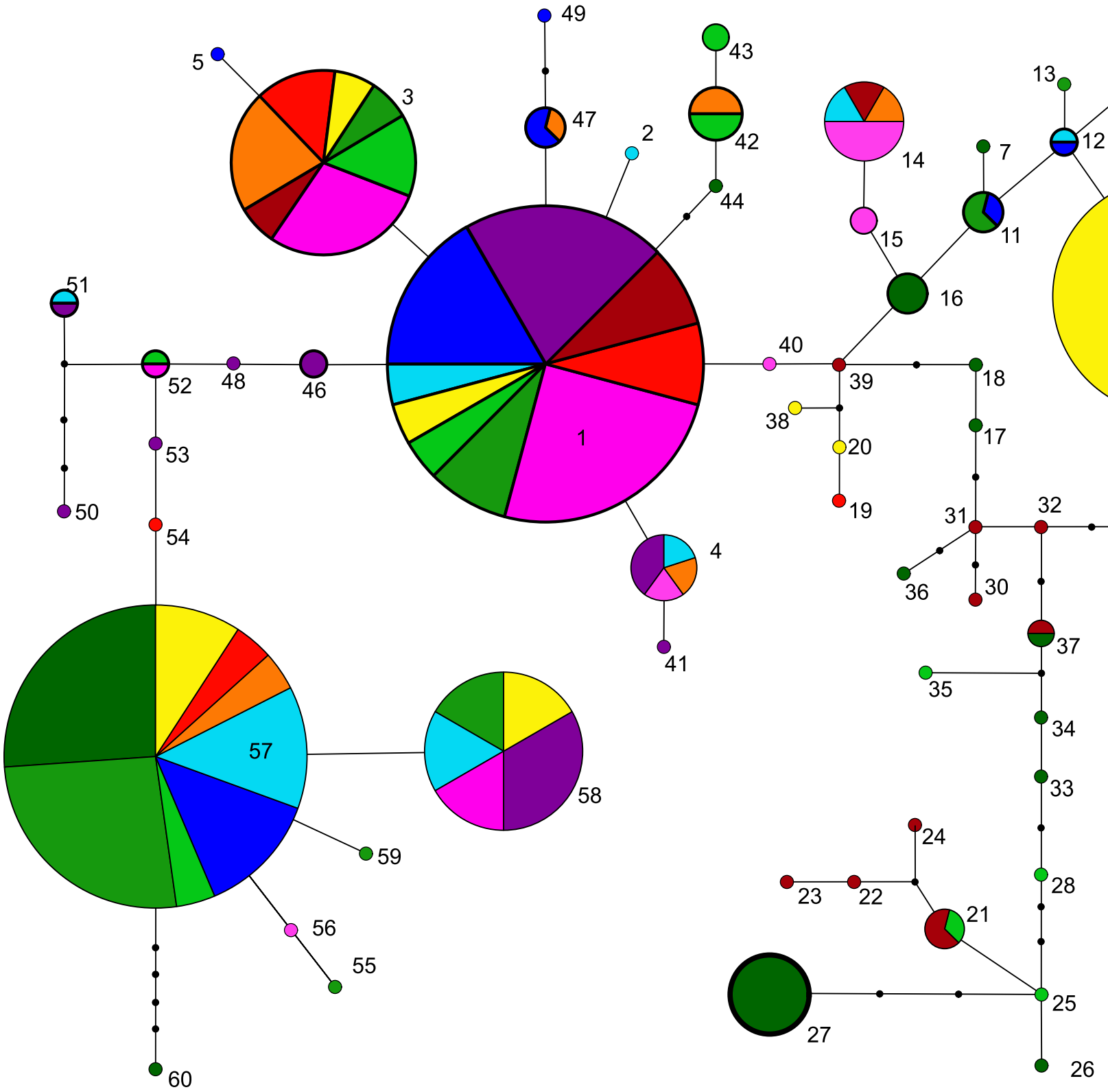
Figure 4. Nested *Vibrio fischeri* haplotype network generated from molecular data acquired in the Philippines during 2010, and 2012 through 2015. Each haplotype is represented by a two-digit identifier (see Fig. 3), with each hierarchical nesting level represented by a 2 to 3 digit dashed identifier and enclosed within dashed and dotted lines. Lines between haplotypes represent the mutational steps required to transition from one genetic station to another, with the small black dots representing un-sampled haplotypes.

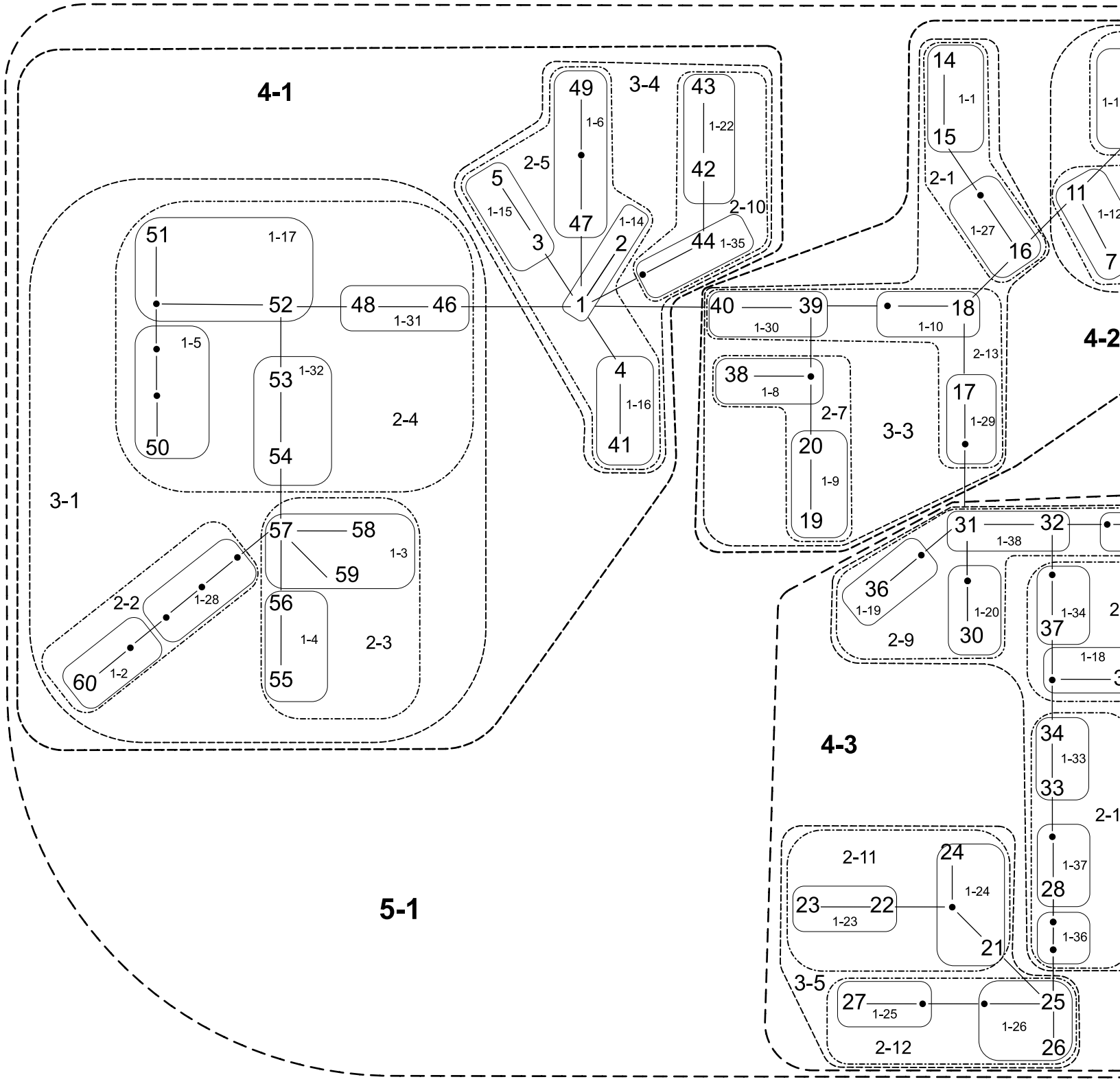
Figure 5. Maps of surface currents in the Philippine islands during: A) Winter and early Spring (Dec-Feb), B) Late Spring (Mar-May) C) Early Summer (June-July), D) Late Summer/ Fall (Aug-Nov). Adapted from Wyrtki 1961.

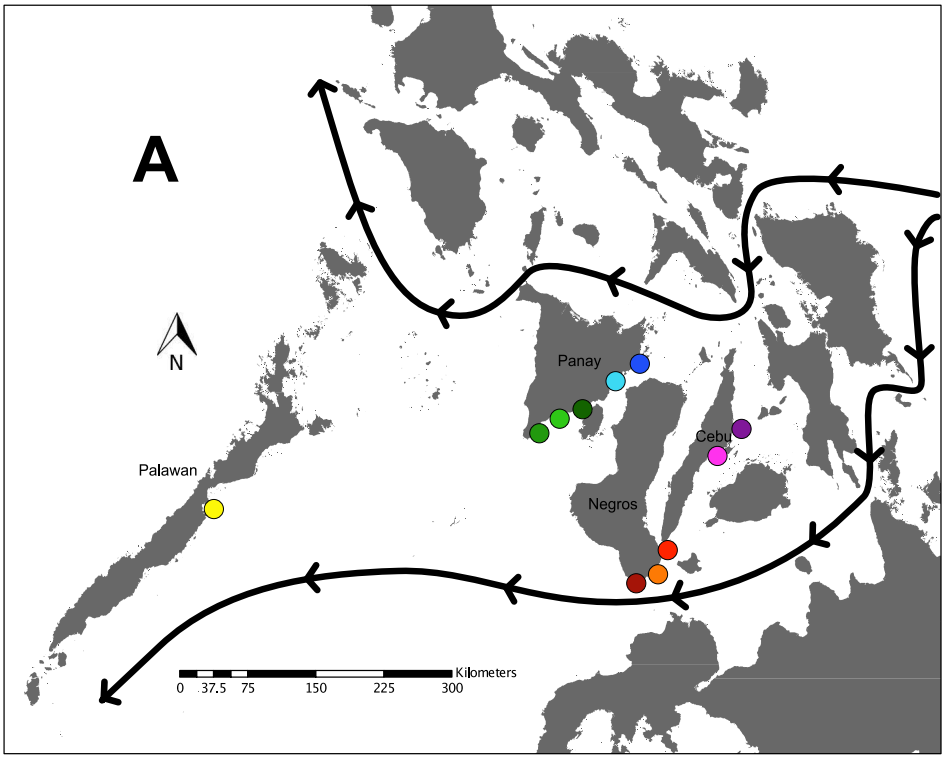

Seafdec
Santo Niño Sur
Parara
San Juan Barotec
Banate
Dumagete
Siliman
Sibulan
Magellan Bay
Cordova
Palawan
Panay
Negros
Cebu
N
0 37.5 75 150 225 300
Kilometers

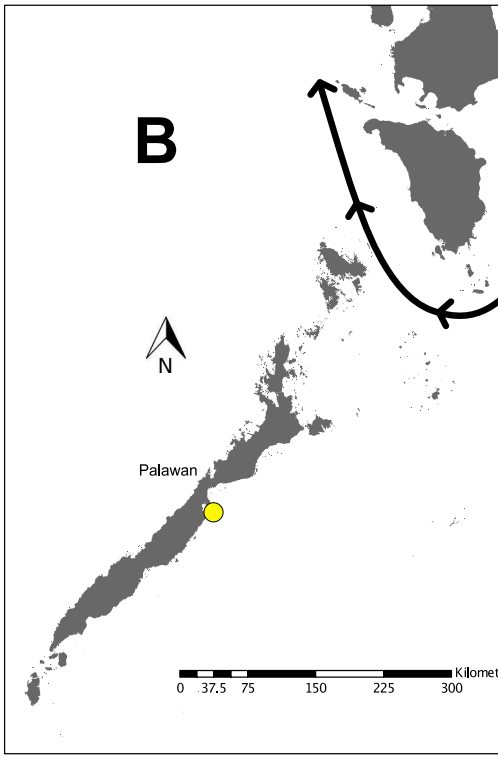

34
20
1-21
18
15
17
1-18
2-11
16
1-19
19
1-17
2-10
3-8
29
25
2-14
23
30
28
26
1-12
27
1-13
2-8
3-6
24
1-11
31
1-14
14
1-20
3-7
22
21
1-16
33
32
1-15
2-9
4-7

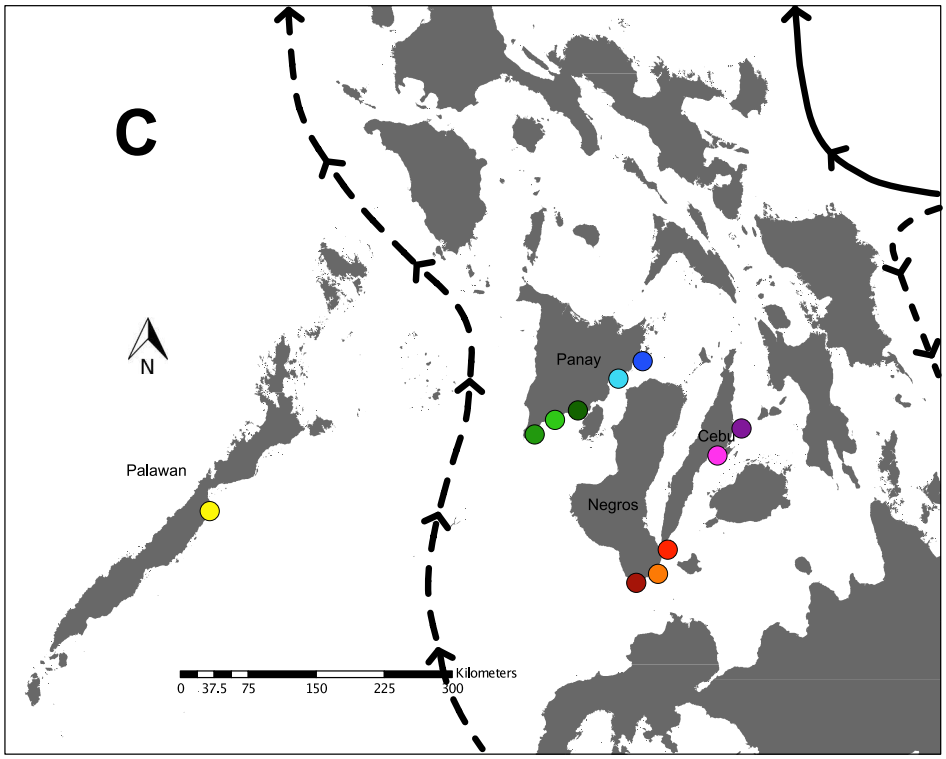

2
1-2
5
6
3
2-2
1
1-3
1-4
4
7
1-5
8
9
1-1
2-1
2-3
11
10
1-6
3-1
4-1

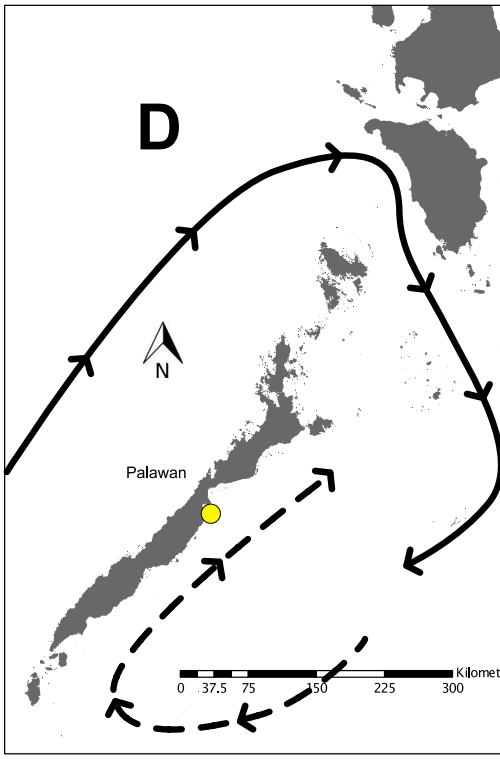

49
5
43
3
47
2
42
44
14
13
7
12
15
11
16
51
40
52
48
46
39
18
38
1
20
17
53
19
50
54
31
32
4
36
30
41
37
35
57
34
58
33
24
59
23
22
28
56
21
55
25
27
60
26

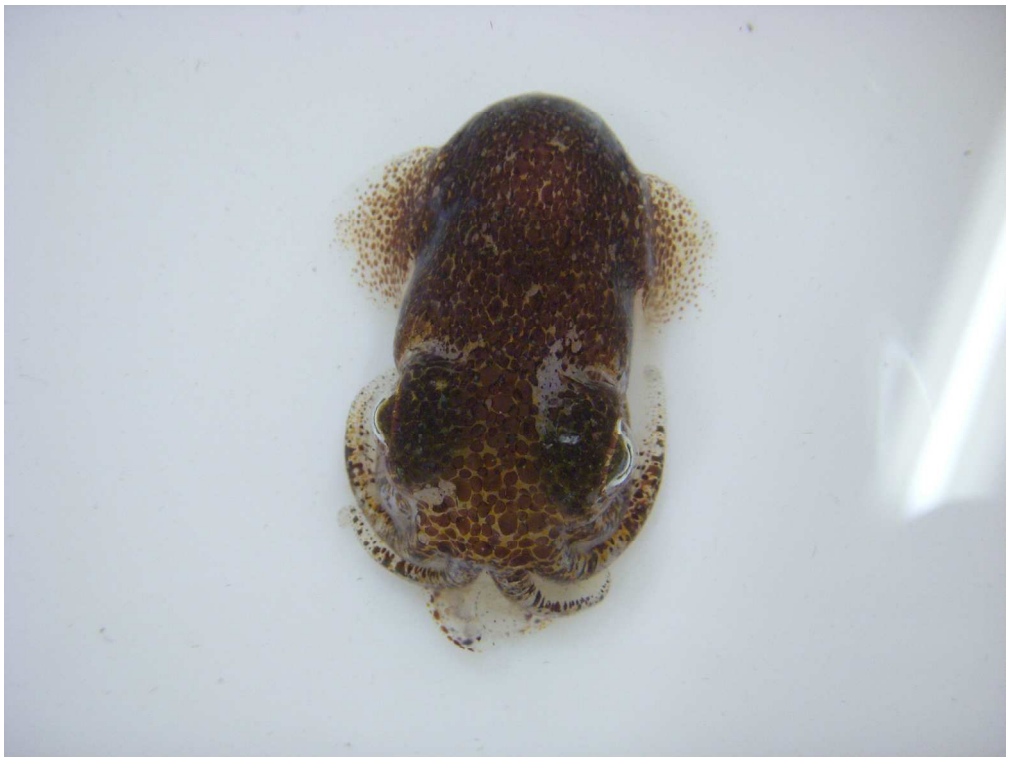

4-1
3-4
49
1-6
43
1-22
2-5
42
5
1-15
47
2-10
1-14
3
2
44
1-35
51
1-17
52
48
46
1-31
1
40
39
1-30
18
1-10
4-2
1-5
53
1-32
4
1-16
38
1-8
2-13
2-4
41
2-7
17
1-29
3-3
50
54
20
1-9
3-1
19
57
58
1-3
59
31
32
1-38
2-2
1-28
56
36
1-19
1-20
1-34
37
2-9
30
1-4
2-3
1-18
60
1-2
55
4-3
34
1-33
33
2-11
24
1-37
1-24
23
22
1-23
28
1-36
21
3-5
27
1-25
25
1-26
2-12
26
5-1


A
N
Panay
Cebu
Palawan
Negros
0 37.5 75 150 225 300
Kilometers


B
N
Palawan
0 37.5 75 150 225 300


C
N
Panay
Cebu
Palawan
Negros
0 37.5 75 150 225 300
Kilometers


D
N
Palawan
0 37.5 75 150 225 300



276x207mm (300 x 300 DPI)

Condensed title: Blood Vessels of the Cephalopod Light Organ

VASCULAR ARCHITECHTURE IN THE BACTERIOGENIC LIGHT ORGAN OF *EUPRYMNA TASMANICA* (CEPHALOPODA: SEPIOLIDAE)

A. J. Patelunas and M. K. Nishiguchi

Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, New Mexico, 88003-8001

Corresponding author: nish@nmsu.edu

Abstract

Symbiosis between Southern dumpling squid, *Euprymna tasmanica* (Cephalopoda: Sepiolidae) and its luminescent symbiont, *Vibrio fischeri*, provides an experimentally tractable system to examine interactions between the eukaryotic host and its bacterial partner. *V. fischeri* luminescence provides light for the squid in a behavior termed "counter-illumination," which allows the squid to mask its shadow amidst downwelling moonlight. Although this association is beneficial, light generated from the bacteria requires large quantities of oxygen to maintain this energy consuming reaction. Therefore, we examined the vascular network within the light organ of juvenile *E. tasmanica* with and without *V. fischeri*. Vessel type, diameter, and location of vessels were measured. Although differences between symbiotic and aposymbiotic squid demonstrated that the presence of *V. fischeri* does not significantly influence the extent of vascular branching at early stages of symbiotic development, these finding do provide an atlas of blood vessel distribution in the organ. Thus, these results provide a framework to understand how beneficial bacteria influence the development of a eukaryotic closed vascular network and provide insight to the evolutionary developmental dynamics that form during mutualistic interactions.

Key words: symbiosis; squid; vasculature; aerobic.

Abbreviations:

Apo= aposymbiotic

Sym= symbiotic

Symbiotic relationships between bacteria and multicellular organisms are very common in nature (Baker 2003; Hirsch and McFall-Ngai 2000; Wang et al. 2011). Beneficial symbioses occur when both members of the association benefit from their interactions with each other. These mutualistic relationships are observed in all major taxa including bacteria, plants, fungi and metazoans (Leigh 2010; Wang et al. 2011; Zamborsky and Nishiguchi 2011). The relationships are highly integrated through their physiologies, metabolic capabilities, and genetic mechanisms that control either the symbiont, or are responsible for host changes to accommodate the symbiont (Bentley et al. 2013; Nadal and Paszkowski 2013; Tschaplinski et al. 2014). Additionally, presence of symbiotic organisms can influence developmental changes in the host, providing evidence of long-term coevolution between the partners (Bergsma and Martinez 2011; Dunlap et al. 2014; Koropatnick et al. 2014). For example, sepiolid squids (Cephalopoda: Sepiolidae) exemplify major morphogenic, biochemical, and physiological changes during infection and colonization by *Vibrio* bacteria from the environment (Foster et al. 2000; Koropatnick et al. 2014; Montgomery and McFall-Ngai 1998). Many of these changes are thought to prevent further colonization by additional environmental symbionts (McFall-Ngai et al. 2010; Nyholm and McFall-Ngai 2004; Nyholm and Nishiguchi 2008; Wier et al. 2010).

Changes in development can be observed and characterized by comparing symbiotic animals to aposymbiotic animals (Claes and Dunlap 2000; Foster et al. 2000; Koropatnick et al.

2004; Sycuro et al. 2006). Extensive vasculature in the light organ of the squid, *Euprymna scolopes,* was observed in earlier studies (McFall-Ngai and Montgomery 1990; Nyholm et al. 2009), but not mapped in detail. Although no hemocytes were tracked in this study, knowledge of how the vasculature develops may provide insight into where hemocytes enter the organ (as previously observed), or how they move to the crypts from the white body where they are produced (Kremer et al. 2014; Nyholm et al. 2009; Schwartzman et al. 2015).

Once infection has occurred by specific *Vibrio* symbionts, the colonized light organ illuminates ventrally to match downwelling moonlight, camouflaging the squid from other benthic organisms during the night. (Jones and Nishiguchi 2004). At dawn, the squid vents 90-95% of the bacteria from the organ into the surrounding seawater, with the remaining 5% re-colonizing the light organ throughout the day. This cycle is repeated daily for the life of the squid. Thus, squids benefit from the bioluminescence of their bacterial symbionts. The host squid in turn provide *Vibrio* bacteria with nutrients, and as a result they grow at least four times as fast in the squid light organ as they do in natural seawater (Lee and Ruby 1994), thereby increasing their overall fitness (Graf and Ruby 2000; Lee and Ruby 1994). While inside the crypts, *V. fischeri* produces light which is regulated by expansion and contraction of the ink sac surrounding the light organ (Jones and Nishiguchi 2004). Production of luminescence via luciferase is aerobically intensive (Goto and Kishi 1968) and may capitalize on oxygen transferred through the squid's circulatory system. Thus, it is plausible that access to oxygen is a limitation to luminescence in the *Euprymna-Vibrio* symbiosis. Since very little is known about whether bacterial colonization induces change in the vasculature of sepiolid squids, the goal of this study was to determine the location and extent of vasculature between symbiotic and aposymbiotic light organs of *E. tasmanica*. Confocal scanning laser microscopy (CSLM) as well

as autofluorescence and Sca*l*e clearing agents (Hama et al. 2011) were used to examine the network of blood vessels in both aposymbiotic and symbiotic squids. The number of branching points (nodes) in each light organ were counted to analyze variation in branching. Diameters of vessels were measured and categorized to determine if there was a clear difference in vasodilation.

Materials and methods

Animal collection and care

Adult *Euprymna tasmanica* were collected from Botany Bay, New South Wales, Australia with permits from the Australian Government, Department of Sustainability, Environment, Water, Population, and Communities (Export permit WT2013-10343), the New South Wales Government, Industry and Investment (Collection permit P04/0014-6.0), and the Australian Government Department of Agriculture, Fisheries, and Forestry Biosecurity (AQIS invoice ELS0016507329). *E. tasmanica* was chosen as the model for this study due to its robustness and ease of rearing in captivity when compared to other species of sepiolids. Animals were transported back to NMSU and kept alive at the NMSU squid facility. Lab raised juvenile *Euprymna tasmanica* were maintained up to 72 hours (3-days) post hatch in 5 mL autoclave sterilized (34 ppt) artificial seawater (Instant Ocean©) in glass scintillation vials (one animal per vial; Nabhitabhata and Nishiguchi, 2014). Symbiotic animals were infected with 5000 colony forming units (CFU) mL^{-1} of *V. fischeri* ETBB 10-1. Animals were maintained with fresh sterilized seawater every 12 hours. Squids were kept on a 12/12 hour light/dark cycle. Colonization of symbiotic animals was confirmed via presence of luminescence in a Berthold Sirius luminometer (Bethold Technologies, Bad Wildbad, Germany).

Animals maintained for longer than 72 hours were raised in 34 ppt autoclave sterilized artificial seawater (Instant Ocean©). One mysid shrimp per squid was placed in a 3 L glass bowl overnight for feeding. Prior to feeding, mysid shrimp were rinsed with autoclaved sterilized DI water, and then maintained in DI water until being placed in the bowl. Any remaining mysid shrimp were removed from the bowl the following morning. Colonization was confirmed via presence of luminescence. For the aposymbiotic tank, 5 μg mL^{-1} of chloramphenicol was added once per week to prevent contamination.

Confocal microscopy

Juvenile squids were appropriately handled with care and under appropriate conditions to minimize any suffering (Nabhitabhata and Nishiguchi 2014). Juveniles were anesthetized on ice for 30 minutes in autoclaved sterilized artificial seawater then fixed with 2.5% glutaraldehyde in 0.1 M imidazole buffer for 48 h. After fixation, animal tissue was initially cleared with Sca*l*eA2 (4 M urea, 10% glycerol, 0.1% Triton X-100) for 2 days, transferred to Sca*l*eB4 (8 M urea, 10% glycerol, 0.1% Triton X-100) for an additional 2 days, and stored in Sca*l*eU2 (4 M urea, 30% glycerol, 0.1% Triton X-100) at room temperature until imaging (Figs. 1A-B; (Hama et al. 2011). Clearing involves making tissue transparent and matching the refractive index throughout the tissue to the refractive index of the immersion media. Mantle cavities were opened from the ventral side and the animal was decapitated directly behind the eyes with a stainless steel razor blade. Whole mantles were opened and placed ventral side down on a 35 mm MatTek glass-bottom dish (MatTek Corporation, Ashland, MA), embedded in 80% glycerol, and covered with a glass coverslip. Dissection, animal orientation, and stereoscopic imaging were completed on a Leica M165FC (Leica Microsystems, Wetzlar, Germany) stereofluorescent microscope.

Autofluorescence was imaged using a laser scanning confocal microscope (Leica TCS SP5 II, Leica Microsystems, Wetzlar, Germany), with a 405 nm UV laser diode, a 488 nm argon-ion laser, and a 561 nm DPSS laser. Autofluorescent spectra for squid tissues were determined by generating λ scans for all available laser lines (Fig. 1C). A 40x 1.25 NA plan-apochromatic oil immersion lens was combined with linear compensation of the photomultiplier tubes (PMT) and acousto-optic tunable filters (AOTF), frame averaging, and stage initiated tiling to generate a high-resolution mosaic of 3 µm optical sections. Blood vessels were identified by comparing region of interest (ROI) emission spectra with known vasculature in the gills (Fig. 2). Different from the surrounding tissue, the ROI spectra inside gill vessels match the ROI spectra inside blood vessels of the light organ. This information was used to infer which pieces of anatomy were blood vessels based on spectra and structural characteristics (tubular, branching, networking structures). Post-processing for confocal images was completed using ImageJ (National Institutes of Health, Bethesda, Maryland, USA) and Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, California, USA).

Morphometric analysis

Diameters were measured manually before and after branch points with the straight-line tool in ImageJ and values were calculated for mean and standard error (SE; (Cartana et al. 2012). Measurements were taken from XY optical sections, and XZ and YZ orthogonal projections. Vessel branches were marked and counted to determine the number of nodes. A minimum of three animals were used for each treatment for biological replicates. All vessels that could be traced were visualized for each animal. Animals were collected and fixed at noon for each of the respective timepoints. A one-way analysis of variance (ANOVA) was used to compare

treatments at the same time point, and the same treatment between time points. *P*-values were corrected with the Holm-Bonferroni test (Holm 1979).

Results

Confocal microscopy

Vessels were divided into four categories based on position relative to the light organ and size: L, the large vessel that runs posterior to anterior; MA, the anterior branch from L going into the lobe; MP, the posterior branch from L going into the lobe; and S, the branches from MA or MP. Lobe refers to one half of the bilobed light organ. Figures 3 and 4 show the position of each of these vessels. L was consistently on the ventral surface of the light organ running proximally posterior to anterior directly under the hindgut on either side. MA and MP form a V branching anterior and posterior, respectively. S vessels were long and branch in no particular pattern. These vessels wrapped around the deep crypts (Figs. 4B, D, F, H, J, L).

Measurements

Comparison by ANOVA of aposymbiotic and symbiotic over three timepoints demonstrated largely insignificant differences, however this analysis provided a descriptive map to the vascular network. The largest trends observed were increases in vessel size at the earliest (1-day) and latest (2-week) timepoints observed. Increases in node number happened between each timepoint as expected during the early stages of development.

In detail: 1-day right lobe L, MA, and MP values are shown in Figure 5 and Table 1 where no differences were observed. Similarly, when 4-day aposymbiotic and symbiotic L, MA, and MP values were evaluated, no differences were found. However, when S measurements were

evaluated for 1-day samples, a significant difference was identified between symbiotic and aposymbiotic animals in the left lobe. Conversely, differences in the number of nodes was observed in the 4-day samples, but not in the 1-day specimens. No major difference was measured in 2-week S vessels, but all other vessels (L, MA, MP) exhibited an increased size in 2-week symbiotic animals.

A comparison of 1-day aposymbiotic and symbiotic left lobes MA and MP were not significantly different (Table 2), but the 1-day L were significantly larger in symbiotic animals. S values of the 1-day specimens were similar to the right lobe. The number of nodes in the left lobe of 1-day samples were not similar.

Four-day samples in the left lobe exhibit no difference between any of the measurements and/or treatments. Comparison of time points within treatments had different results. When the 1-day aposymbiotic animal lobes were compared with the 4-day aposymbiotic animal lobes, MA diameter was not significantly different (P= 0.13 for R and P= 0.95 for L) but the number of nodes were significantly greater in the right lobe. Changes from 1-day symbiotic animal S vessels to 4-day symbiotic animal S vessels were also significant (P= 0.0003 for R and P =0.003 for L) demonstrating fast growth.

When comparing the 2-week specimens, a significant increase in size was observed in the symbiotic L vessels when compared with the aposymbiotic. Most interestingly were the changes from the 4-day samples to the 2-week samples within treatment. Aposymbiotic squid exhibited no significant changes in the number of nodes (P= 0.09 for R and 0.04 for L), but symbiotic animals significantly increased the number of nodes (P= 0.00001 for R and P= 0.003 for L). No other significant differences were consistent between lobes.

Discussion

Oxygen delivery to *V. fischeri* symbionts inhabiting the light organ is crucial for understanding the biochemical reactions that produce luminescence that are necessary for a successful mutualism. Therefore, this study examined the extent of vascularization in the developing bacteriogenic light organ of *E. tasmanica* during early post-hatching development. Previous studies have demonstrated the presence of the blood vessels in adult squids (McFall-Ngai and Montgomery 1990; Nyholm et al. 2009) as well as anaerobic conditions inside the light organ (Ruby and McFall-Ngai 1999; Schwartzman et al. 2015; Wier et al. 2010). These studies provide evidence that the presence of *V. fischeri* in the light organ does not influence angiogenesis. Oxygen is required for the catalysis of luciferase during bioluminescence in *V. fischeri* (Goto 1968), and therefore needs to be present in large quantities to sustain bioluminescence in addition to aerobic metabolism of both *V. fischeri* symbionts and their host squid.

Although the number of branching events does not appear to be significantly different between aposymbiotic and symbiotic animals, there is significance between S vessel diameter in the 1-day sample's left lobe (Fig. 5; Table 1). Within the first 24 hours after inoculation, cell death occurs in the appendages and surface epithelial cells of the light organ (Foster et al. 2000; Koropatnick et al. 2014). Cells directly in contact with *V. fischeri* further differentiate, becoming cuboidal and increasing size (Lamarcq and McFall-Ngai 1998; Montgomery and McFall-Ngai 1994; Sycuro et al. 2006). Since this is a vital time point for symbiont initiation, vasodilation might be initiated by *V. fischeri*. Nitric oxide (NO), which is known to be a vasodilator, is released during the early stages of the symbiosis as a defense against non-symbiotic bacteria accumulation. However, NO is shown to steadily decrease after the initial inoculation of bacteria

(Davidson et al. 2004). In this study, the 4-day time point demonstrated no significant difference between any of the vessel diameters. Blood vessels will return to a resting diameter with a reduction in NO instead of being dilated as observed in 1-day symbiotic samples. This suggests that after symbiont establishment, delivery of substances in the hemolymph (nutrients, oxygen, hemocytes, *etc*.) is reduced to a pre-symbiotic state, similar to that of aposymbiotic animals. Alternatively, this difference could simply be an artifact of low sample size (n=3 for each set). Observations of how these vessels change with concentration of *V. fischeri* during the height of their infection (at night) and pre and post-venting (pre and post dawn), might give additional insight as to how specific vasodialators control size during different stages of the mutualism.

The lack of any difference in the number of nodes in the 1-day samples might be a result of developmental time. Angiogenesis induced by low-oxygen requires continual anaerobic stress (Shweiki et al. 1992). If anaerobic stress is induced by the presence of *V. fischeri*, it would be during the period of highest oxygen demand, or during luminescence. This period only lasts a maximum of ~12 hours during the night when *V. fischeri* are at their highest concentrations. In a 1-day sample, ~12 hours is too short of a period to generate differences in angiogenesis. Longer time points were examined to determine if developmental timing was influential on vascular growth (Fig. 4 E-L). A significant difference was found in the number of nodes for 4-day right lobes, but not for left lobes. This difference between lobes may be due to the low number of samples examined for each time point (n=3). *P* values for 4-day samples were vastly different. However, this is not the only measurement that exhibited such a difference between lobes.

Potential differences between the right and left lobe can be explained by asymmetric development in protostomes. Although it has not been extensively explored, left-right (LR) asymmetry during development has been observed in nematodes and pulmonate snails (Okumura

et al. 2008). Mollusca as a group has evolved and lost symmetry numerous times through evolutionary history. Among cephalopods, the conversion from symmetry to asymmetry is estimated to have changed at least five times with right and left bias evolving simultaneously (Palmer 1996). Light organs of the related congener *Semirossia* also share asymmetry in the ducts leading to the light organ (Boletzky 1970). This similarity may provide indirect evidence for the onset of morphological changes that induced development of the original light organ present in basal sepiolid squids (such as *Semirossia*), and lends the developmental foundation for a haven for bacteria, such as *V. fischeri*, to initiate a beneficial association (Nishiguchi *et al*., 2004; Naef, 1921).

Imaging of juvenile squids shows the posterior aorta branching to feed blood into the light organ. This pathway has been previously observed in *Semirossia tenera* (Boletzky, 1970). Afferent vessels enter the light organ on the ventral side (Fig. 3 and 4A-L) to either side of the hindgut. Our study confirms the vascular anatomical similarities in light organs between distantly related symbiotic squid (Nishiguchi et al. 2004). A decreased node number or lack of change in vessel diameter may compensate oxygen delivery by host squids elevating their heart rate. Future projects might investigate the rate or volume output of the systemic heart, since it may be an alternative way to deliver an increased supply of oxygen and nutrients to the light organ. Additionally, vessel diameter can be examined at varying *in vivo* time points to determine whether the diameter changes over time are due to the daily metabolic activity of the squids.

By examining morphological changes between aposymbiotic and symbiotic sepiolid squids, this study has provided further insights of how beneficial mutualisms are sustained. Additionally, by understanding the location and extent of vascular anatomy in the light organ, the role of hemocyte migration into the crypts can be tracked and analyzed. Other symbiotic systems

have demonstrated nutrient exchange between symbiotic partners (Baker 2003; Denison and Kiers 2011), and only recently has oxygen transfer in the sepiolid squid begun to be explored (Kremer et al. 2014). These results demonstrate that the *E. tasmanica* circulatory system develops the same in both symbiotic and aposymbiotic juveniles, regardless of *V. fischeri* colonization; however, the functional changes in the presence of *V. fischeri* remain to be determined. Live confirmation of vasodilation in the early stages of the symbiosis would provide additional information regarding the developmental ontogeny in a dependent aerobic system located in a dynamic beneficial association. A thorough understanding of how the vascular system adapts under symbiotic stress provides a mechanism for testing the innate immune response on an organismal scale, and an alternative hypothesis for the role of a circulatory system in maintaining beneficial associations such as vertebrate gut microbes.

Acknowledgements

The authors would like to thank P. H. Cook at the NMSU CURRL facility for help with all microscopy. This work was supported by NIH NIAID 1SC1AI081659, NIH NIAIDS 3SC1AI081659-02S1, and NSF IOS 074498 to M.K.N. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Biosafety Committee of New Mexico State University (Permit Number: 1306NMD20103) and under the guidelines of the NMSU's Institutional Animal Care and Use Committee (85-R-009 and OLAW A4022-01 and IACUC license 2013-029).

References

Baker AC. 2003. Flexibility and specificity in coral-algal symbiosis: Diversity, ecology, and biogeography of *Symbiodinium*. Annu. Rev. Ecol. Evol. Syst. 34:661-689.

Bentley K, Jones M & Cruys B. 2013. Predicting the future: Towards symbiotic computational and experimental angiogenesis research. Exp. Cell Res. 319:1240-1246.

Bergsma GS & Martinez CM. 2011. Mutualist-induced morphological changes enhance growth and survival of corals. Mar. Biol. 158:2267-2277.

Boettcher KJ & Ruby EG. 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. J. Bacteriol. 177:1053-8.

Boletzky SV. 1970. On Presence of Light Organs in *Semirossia* Steenstrup, 1887 (Mollusca - Cephalopoda). Bull. Mar. Sci. 20:374-&.

Cartana T, Saftoiu A, Gruionu LG, Gheonea DI, Pirici D, Georgescu CV, Ciocalteu A & Gruionu G. 2012. Confocal Laser Endomicroscopy for the Morphometric Evaluation of Microvessels in Human Colorectal Cancer Using Targeted Anti-CS1 Antibodies. PLoS One 7.

Claes MF & Dunlap PV. 2000. Aposymbiotic culture of the sepiolid squid *Euprymna scolopes*: Role of the symbiotic bacterium *Vibrio fischeri* in host animal growth, development, and light organ morphogenesis. J. Exp. Zool. 286:280-296.

Davidson SK, Koropatnick TA, Kossmehl R, Sycuro L & McFall-Ngai MJ. 2004. NO means 'yes' in the squid-vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. Cell Microbiol. 6:1139-1151.

Denison RF & Kiers ET. 2011. Life Histories of Symbiotic Rhizobia and Mycorrhizal Fungi. Curr. Biol. 21:R775-R785.

Dunlap PV, Takami M, Wakatsuki S, Hendry TA, Sezaki K & Fukui A. 2014. Inception of bioluminescent symbiosis in early developmental stages of the deep-sea fish, *Coelorinchus kishinouyei* (Gadiformes: Macrouridae). Ichthyol. Res. 61:59-67.

Foster JS, Apicella MA & McFall-Ngai MJ. 2000. *Vibrio fischeri* lipopolysaccharide induces developmental apoptosis, but not complete morphogenesis, of the *Euprymna scolopes* symbiotic light organ. Dev. Biol. 226:242-254.

Goto T. 1968. Chemistry of Bioluminescence. Pure Appl. Chem 17:421-442.

Goto T & Kishi Y. 1968. Luciferins Bioluminescent Substances. Angew. Chem.-Int. Edit. 7:407-&.

Graf J & Ruby EG. 2000. Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. Mol. Microbiol. 37:168-179.

Hama H, Kurokawa H, Kawano H, Ando R, Shimogori T, Noda H, Fukami K, Sakaue-Sawano A & Miyawaki A. 2011. Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. Nat. Neurosci. 14:1481-U166.

Hirsch AM & McFall-Ngai MJ. 2000. Fundamental concepts in symbiotic interactions: Light and dark, day and night, squid and legume. J. Plant Growth Regul. 19:113-130.

Holm S. 1979. A Simple Sequentially Rejective Multiple Test Procedure. Scand. J. Stat. 6:65-70.

Intaglietta M, Johnson PC & Winslow RM. 1996. Microvascular and tissue oxygen distribution. Cardio. Re. 32:632-643.

Jones BW & Nishiguchi MK. 2004. Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes*; Berry (Mollusca: Cephalopoda). Mar. Biol. 144:1151-1155.

Koropatnick T, Goodson MS, Heath-Heckman EA & McFall-Ngai M. 2014. Identifying the cellular mechanisms of symbiont-induced epithelial morphogenesis in the squid-*Vibrio* association. Biol Bull 226:56-68.

Koropatnick TA, Engle JT, Apicella MA, Stabb EV, Goldman WE & McFall-Ngai MJ. 2004. Microbial factor-mediated development in a host-bacterial mutualism. Science 306:1186-1188.

Kremer N, Schwartzman J, Augustin R, Zhou L, Ruby EG, Hourdez S & McFall-Ngai MJ. 2014. The dual nature of haemocyanin in the establishment and persistence of the squid-vibrio symbiosis. Proc. Biol. Sci. Roy. Soc. 281:20140504.

Lamarcq LH & McFall-Ngai MJ. 1998. Induction of a gradual, reversible morphogenesis of its host's epithelial brush border by *Vibrio fischeri*. Infect. Immun. 66:777-785.

Lee KH & Ruby EG. 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl. Environ. Microbiol. 60:1565-1571.

Leigh EG. 2010. The evolution of mutualism. J. Evol. Biol. 23:2507-2528.

McFall-Ngai M & Montgomery MK. 1990. The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda, Sepiolidae). Biol. Bull. 179:332-339.

McFall-Ngai M, Nyholm SV & Castillo MG. 2010. The role of the immune system in the initiation and persistence of the *Euprymna scolopes-Vibrio fischeri* symbiosis. Sem. Immunol. 22:48-53.

Montgomery MK & McFall-Ngai M. 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Eurpymna scolopes*. Development 120:1719-1729.

Montgomery MK & McFall-Ngai MJ. 1998. Late postembryonic development of the symbiotic light organ of *Euprymna scolopes* (Cephalopoda : Sepiolidae). Biol. Bull. 195:326-336.

Nabhitabhata J & Nishiguchi MK. 2014. *Euprymna hyllebergi* and *Euprymna tasmanica*. In: Cephalopod Culture. Iglesias J, Fuentes L, Villanueva R, eds. pp. 253-269. Springer Netherlands.

Nadal M & Paszkowski U. 2013. Polyphony in the rhizosphere: presymbiotic communication in arbuscular mycorrhizal symbiosis. Curr. Opin. Plant Biol. 16:473-479.

Nishiguchi MK, Lopez JE & von Boletzky S. 2004. Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. Evol. Dev. 6:41-49.

Nyholm SV & McFall-Ngai MJ. 2004. The winnowing: Establishing the squid-*Vibrio* symbiosis. Nat. Rev. Microbiol. 2:632-642.

Nyholm SV & Nishiguchi MK. 2008. The evolutionary ecology of a Sepiolid squid-*Vibrio* association: from cell to environment. Vie Et Milieu-Life Environ. 58:175-184.

Nyholm SV, Stewart JJ, Ruby EG & McFall-Ngai MJ. 2009. Recognition between symbiotic *Vibrio fischeri* and the haemocytes of *Euprymna scolopes*. Environ. Microbiol. 11:483-493.

Okumura T, Utsuno H, Kuroda J, Gittenberger E, Asami T & Matsuno K. 2008. The development and evolution of left-right asymmetry in invertebrates: Lessons from *Drosophila* and snails. Dev. Dyn. 237:3497-3515.

Palmer AR. 1996. From symmetry to asymmetry: Phylogenetic patterns of asymmetry variation in animals and their evolutionary significance. Proc. Natl. Acad. Sci. U. S. A. 93:14279-14286.

Ruby EG & McFall-Ngai MJ. 1999. Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. Trends Microbiol. 7:414-420.

Schwartzman JA, Koch E, Heath-Heckman EAC, Zhou L, Kremer N, McFall-Ngai MJ & Ruby EG. 2015. The chemistry of negotiation: Rhythmic, glycan-driven acidification in a symbiotic conversation. Proc. Nat. Acad. Sci. 112:566-571.

Shweiki D, Itin A, Soffer D & Keshet E. 1992. Vascular endothelial growth-factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843-845.

Sycuro LK, Ruby EG & McFall-Ngai M. 2006. Confocal microscopy of the light organ crypts in juvenile *Euprymna scolopes* reveals their morphological complexity and dynamic function in symbiosis. J. Morphol. 267:555-568.

Tschaplinski TJ, Plett JM, Engle NL, Deveau A, Cushman KC, Martin MZ, Doktycz MJ, Tuskan GA, Brun A, Kohler A & Martin F. 2014. *Populus trichocarpa* and *Populus deltoides* exhibit different metabolomic responses to colonization by the symbiotic fungus *Laccaria bicolor*. Mol. plant-microbe interact. 27:546-56.

Wang XR, Pan QA, Chen FX, Yan XL & Liao H. 2011. Effects of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on soybean growth as related to root architecture and availability of N and P. Mycorrhiza 21:173-181.

Wier AM, Nyholm SV, Mandel MJ, Massengo-Tiasse RP, Schaefer AL, Koroleva I, Splinter-BonDurant S, Brown B, Manzella L, Snir E, Almabrazi H, Scheetz TE, Bonaldo MD, Casavant TL, Soares MB, Cronan JE, Reed JL, Ruby EG & McFall-Ngai MJ. 2010. Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. Proc. Natl. Acad. Sci. U. S. A. 107:2259-2264.

Zamborsky DJ & Nishiguchi MK. 2011. Phylogeographical patterns among Mediterranean sepiolid squids and their *Vibrio* symbionts: Environment drives specificity among sympatric species. Appl. Environ. Microbiol. 77:642-649.

Figure Legends

Figure 1. Bright field imaging of a fixed and fixed then Sca*l*e cleared 24 hour juvenile *E. tasmanica* light organs and emission spectra for Sca*l*e cleared mantle tissue. **A**. Ventral view of an untreated juvenile light organ removed from the squid mantle cavity. Anterior portion of the animal is towards the top of the figure. **B**. Ventral view of a Sca*l*e cleared juvenile light organ removed from the squid mantle cavity. Anterior portion of the animal is towards the top of the figure. **C.** Emission spectra controls for squid muscle tissue exhibiting normalized mean intensity (y-axis) over wavelength in nm (x-axis). The laser line used for the spectrum is in the top right corner of each graph. Scale bar = 500 µm.

Figure 2. Region of interest (ROI) λ scans of blood vessels in the gills (A) and in the light organ (B) of a 12 day old *E. tasmanica*. **A**. XY optical section of the squid gill with the 405 nm laser. The white box indicates the ROI scanned for the emission spectrum. **B**. XY optical section of the central tissue in the light organ with the 405 nm laser. The white box represents the ROI in which the emission spectrum was measured. **C.** Emission spectra for scans in A (black line) and B (grey line). Scale bar = 100 µm.

Figure 3. A diagram proposing the orientation and hierarchy of blood vessels throughout the light organ as described in this paper. Posterior is to the top. SH= systemic heart; MA= mantle; GL= gill; LO= light organ; L= the largest vessel that bifurcates to each lobe of the light organ; MP= the posterior branch of the second tier of vessels; MA= the anterior branch of the second tier of vessels; S= the third tier of vessels that sprawls throughout the organ.

Figure 4. Optical sections of each light organ treatment observed**.** Light organs are oriented from a ventral view with posterior at the top. All images are a merger of laser lines 405 nm (blue), 488 nm (green), and 561 nm (red). Top column represents shallow optical section of the surface of the light organ and large vessel. Deep column represents the deep crypts and associated vessels. **A-D**. 1-Day aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. **E-H**. 4-Day aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. **I-L.** 2-Week aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. ap= anterior appendage; po= pore; cc= crypt Scale bar = 100 µm.

Figure 5. Vessels diameters in µm of light organ lobes comparing aposymbiotic and symbiotic animals in average ± standard error. Columns labeled at the top for the respective lobe. **A and F.** Largest vessel (L) diameters of 1-Day, 4-Day, and 2-week animals. **B and G.** MP diameters of 1-Day, 4-Day, and 2-week animals. **C and H.** MA diameters of 1-Day, 4-Day, and 2-week animals. **D and I.** Smallest vessel (S) diameters of 1-Day, 4-Day, and 2-week animals. **E and J.** Number of branch points (nodes) counted for each of the samples. Significance of $P < 0.5$ is indicated by asterisk (*).

Table 1. Measurements of each lobe for aposymbiotic and symbiotic juvenile squids. L= large vessels, MA= medium anterior vessels; MP=medium posterior vessels; S= small vessels

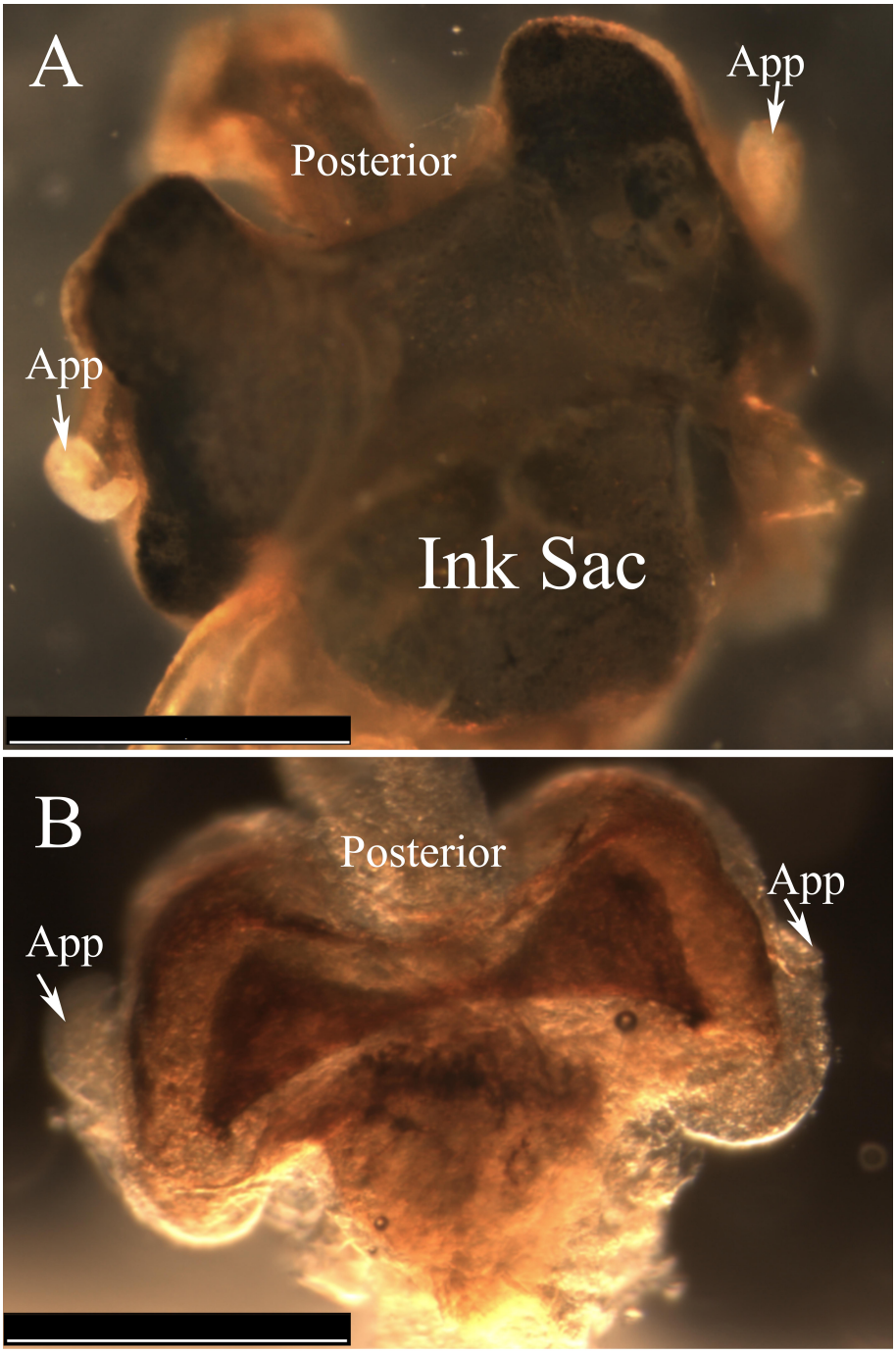
Diameters of blood vessels (µm)						
Right Lobe						
	1 day apo	**1 day sym**	**4 day apo**	**4 day sym**	**2 week apo**	**2 week sym**
N	3	3	3	4	3	3
L	30.02 ± 3.42	31.83 ± 4.32	29.46 ± 0.21	26.71 ± 2.32	20.92 ± 8.08	41.40 ± 2.89
MA	14.23 ± 0.11	11.97 ± 1.60	9.69 ± 3.12	9.71 ± 2.21	11.06 ± 1.91	14.98 ± 0.24
MP	14.05 ± 2.35	12.71 ± 0.87	9.95 ± 1.15	10.45 ± 0.84	11.78 ± 2.39	12.33 ± 2.22
S	5.17 ± 0.66	6.56 ± 0.40	4.75 ± 0.25	4.23 ± 0.23	4.15 ± 0.15	4.40 ± 0.16
Nodes	10.00 ± 0.18	8.33 ± 0.88	12.00 ± 0.58	8.25 ± 0.25	18.67 ± 2.91	17.67 ± 0.21
Left Lobe						
	1 day apo	**1 day sym**	**4 day apo**	**4 day sym**	**2 week apo**	**2 week sym**
N	3	3	3	4	3	3
L	24.28 ± 5.14	36.56 ± 3.85	28.38 ± 2.95	26.93 ± 3.24	30.12 ± 1.31	36.57 ± 5.36
MA	11.23 ± 1.05	12.15 ± 1.04	11.62 ± 1.65	8.82 ± 1.51	12.19 ± 1.56	11.37 ± 1.49
MP	13.12 ± 1.60	14.05 ± 1.82	11.06 ± 1.74	11.15 ± 1.16	10.46 ± 1.82	11.61 ± 2.07
S	4.59 ± 0.26	6.22 ± 0.51	4.02 ± 0.23	4.29 ± 0.25	3.97 ± 0.18	4.13 ± 0.17
Nodes	8.33 ± 0.23	9.33 ± 0.33	10.33 ± 1.20	10.00 ± 0.91	18.67 ± 2.60	18.33 ± 0.31

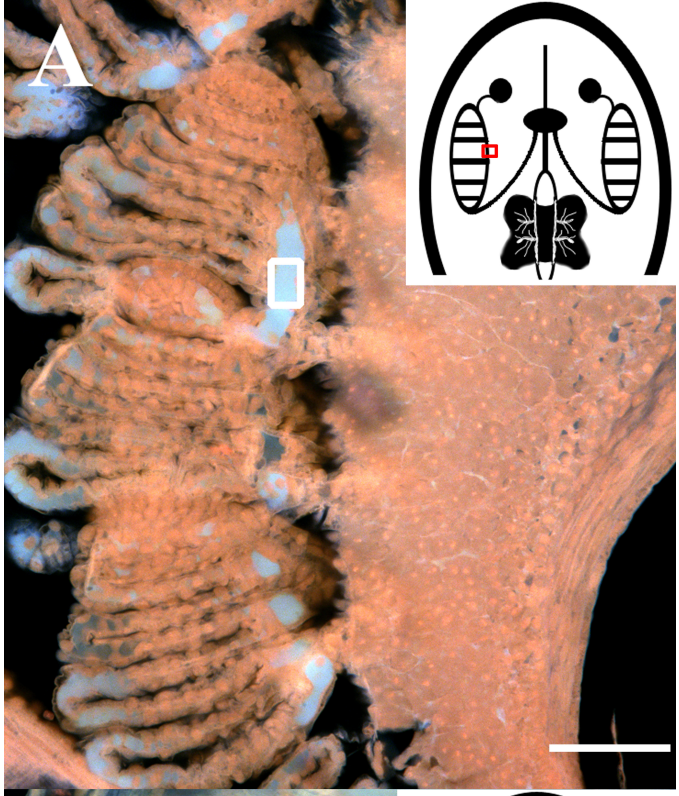
Table 2. *P* values designating comparison between aposymbiotic and symbiotic vessel sizes. Significant values are indicated by bolding and asterisk. L= large vessels, MA= medium anterior vessels; MP=medium posterior vessels; S= small vessels

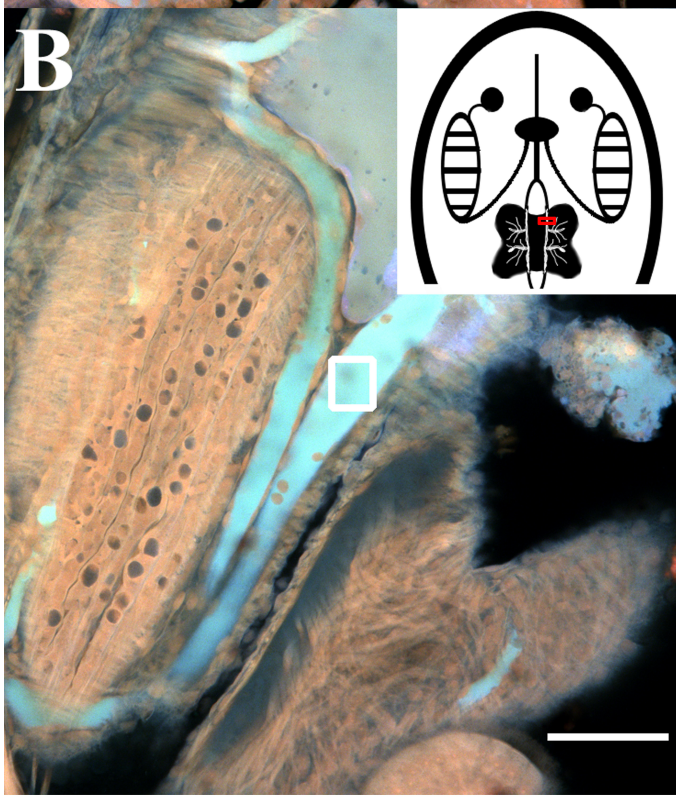
Right Lobe				Left Lobe			
Vessel	**Condition and Time**	**Compared to**	***P***	**Vessel**	**Condition and Time**	**Compared to**	***P***
L	1-Day Sym	4-Day Sym	0.31	L	1-Day Sym	4-Day Sym	0.11
L	1-Day Sym	1-Day Apo	0.76	L	1-Day Sym	1-Day Apo	0.13
L	4-Day Sym	2-Week Sym	**0.01***	L	4-Day Sym	2-Week Sym	0.16
L	4-Day Sym	4-Day Apo	0.36	L	4-Day Sym	4-Day Apo	0.76
L	2-Week Sym	2-Week Apo	**0.01***	L	2-Week Sym	2-Week Apo	0.31
L	1-Day Apo	4-Day Apo	0.88	L	1-Day Apo	4-Day Apo	0.52
L	4-Day Apo	2-Week Apo	0.13	L	4-Day Apo	2-Week Apo	0.62
MA	1-Day Sym	4-Day Sym	0.52	MA	1-Day Sym	4-Day Sym	0.15
MA	1-Day Sym	1-Day Apo	0.23	MA	1-Day Sym	1-Day Apo	0.57
MA	4-Day Sym	2-Week Sym	0.49	MA	4-Day Sym	2-Week Sym	0.81
MA	4-Day Sym	4-Day Apo	0.93	MA	4-Day Sym	4-Day Apo	0.27
MA	2-Week Sym	2-Week Apo	0.69	MA	2-Week Sym	2-Week Apo	0.84
MA	1-Day Apo	4-Day Apo	**0.05***	MA	1-Day Apo	4-Day Apo	0.85
MA	4-Day Apo	2-Week Apo	0.62	MA	4-Day Apo	2-Week Apo	0.31
MP	1-Day Sym	4-Day Sym	0.5	MP	1-Day Sym	4-Day Sym	0.22
MP	1-Day Sym	1-Day Apo	0.62	MP	1-Day Sym	1-Day Apo	0.72
MP	4-Day Sym	2-Week Sym	0.06	MP	4-Day Sym	2-Week Sym	0.82
MP	4-Day Sym	4-Day Apo	0.4	MP	4-Day Sym	4-Day Apo	0.96
MP	2-Week Sym	2-Week Apo	0.25	MP	2-Week Sym	2-Week Apo	0.72
MP	1-Day Apo	4-Day Apo	0.19	MP	1-Day Apo	4-Day Apo	0.43
MP	4-Day Apo	2-Week Apo	0.53	MP	4-Day Apo	2-Week Apo	0.75
S	1-Day Sym	4-Day Sym	**0.0003***	S	1-Day Sym	4-Day Sym	**0.001***
S	1-Day Sym	1-Day Apo	0.06	S	1-Day Sym	1-Day Apo	**0.02***
S	4-Day Sym	2-Week Sym	0.52	S	4-Day Sym	2-Week Sym	0.97
S	4-Day Sym	4-Day Apo	0.13	S	4-Day Sym	4-Day Apo	0.45
S	2-Week Sym	2-Week Apo	0.24	S	2-Week Sym	2-Week Apo	0.62
S	1-Day Apo	4-Day Apo	0.71	S	1-Day Apo	4-Day Apo	0.12
S	4-Day Apo	2-Week Apo	**0.03***	S	4-Day Apo	2-Week Apo	0.56
Nodes	1-Day Sym	4-Day Sym	0.85	Nodes	1-Day Sym	4-Day Sym	0.58
Nodes	1-Day Sym	1-Day Apo	0.19	Nodes	1-Day Sym	1-Day Apo	0.25
Nodes	4-Day Sym	2-Week Sym	**0.00001***	Nodes	4-Day Sym	2-Week Sym	**0.04***
Nodes	4-Day Sym	4-Day Apo	**0.0003***	Nodes	4-Day Sym	4-Day Apo	0.83
Nodes	2-Week Sym	2-Week Apo	0.76	Nodes	2-Week Sym	2-Week Apo	0.91
Nodes	1-Day Apo	4-Day Apo	0.07	Nodes	1-Day Apo	4-Day Apo	0.22
Nodes	4-Day Apo	2-Week Apo	0.09	Nodes	4-Day Apo	2-Week Apo	**0.003***

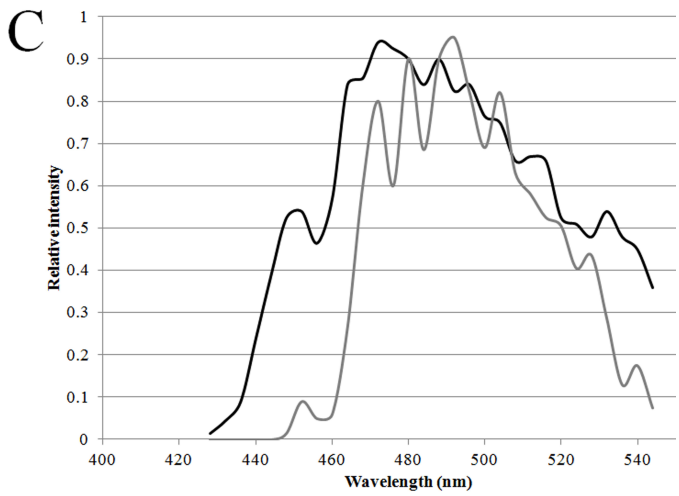
469

470


A
App
Posterior
App
Ink Sac
B
Posterior
App
App
C
1.0
0.8
0.6
0.4
0.2
0
Mean Intensity
400
500
700
800
Wavelength (nm)
405 nm
458 nm
488 nm
514 nm
561 nm
633 nm


A


B


C
Relative intensity
Wavelength (nm)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
400
420
440
460
480
500
520
540

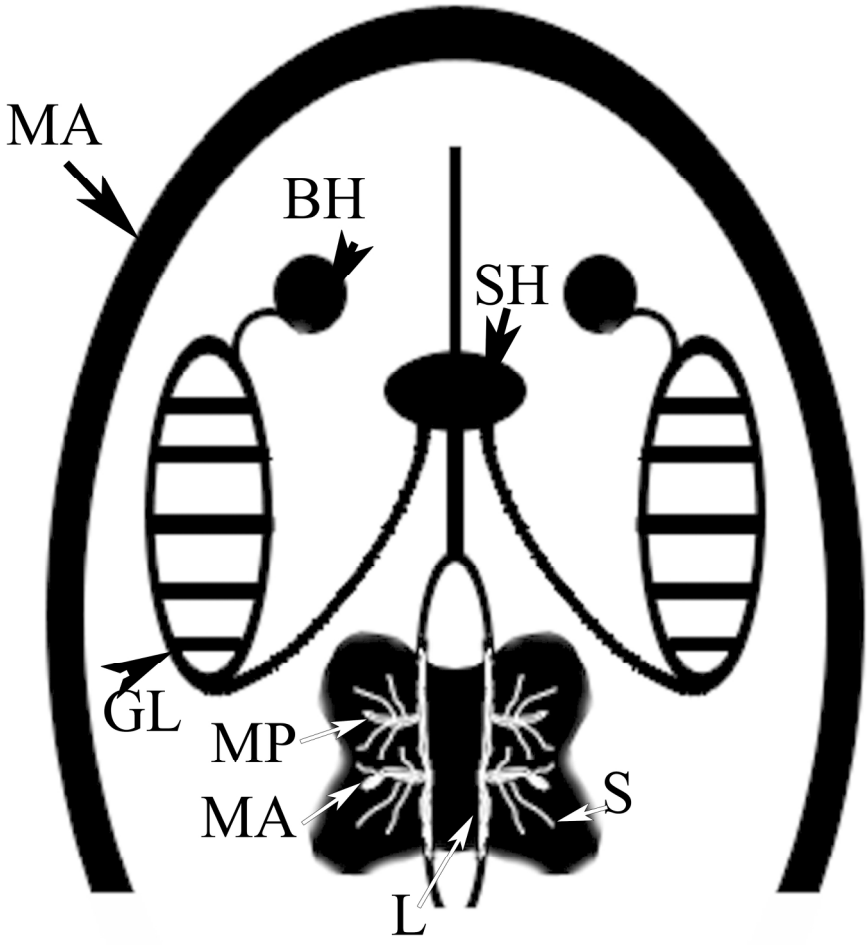


Figure 3. A diagram proposing the orientation and hierarchy of blood vessels throughout the light organ as described in this paper. Posterior is to the top. SH= systemic heart; MA= mantle; GL= gill; LO= light organ; L= the largest vessel that bifurcates to each lobe of the light organ; MP= the posterior branch of the second tier of vessels; MA= the anterior branch of the second tier of vessels; S= the third tier of vessels that sprawls throughout the organ.

96x93mm (600 x 600 DPI)

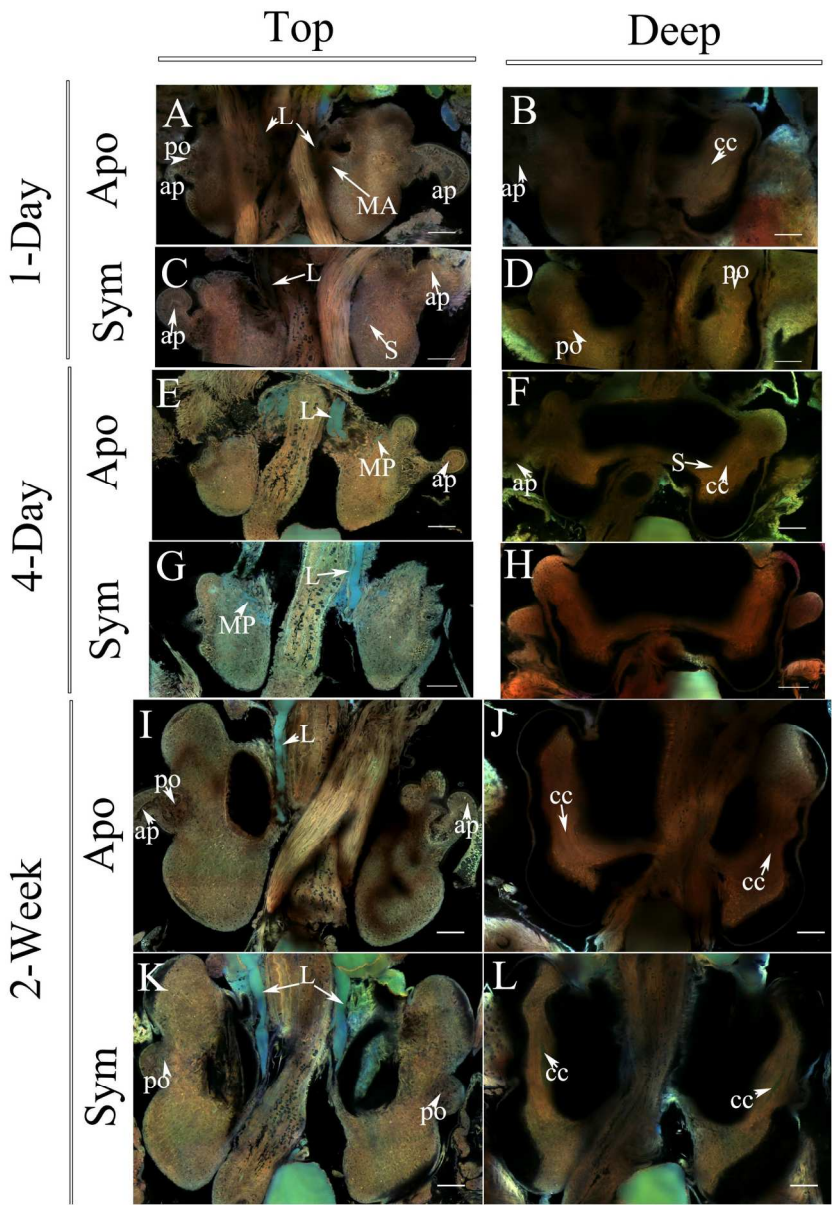


Figure 4. Optical sections of each light organ treatment observed. Light organs are oriented from a ventral view with posterior at the top. All images are a merger of laser lines 405 nm (blue), 488 nm (green), and 561 nm (red). Top column represents shallow optical section of the surface of the light organ and large vessel. Deep column represents the deep crypts and associated vessels. A-D. 1-Day aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. E-H. 4-Day aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. I-L. 2-Week aposymbiotic (A-B) and symbiotic (C-D) samples exhibiting light organ surface and deep crypts. ap= anterior appendage; po= pore; cc= crypt Scale bar = 100 μm.

229x323mm (300 x 300 DPI)

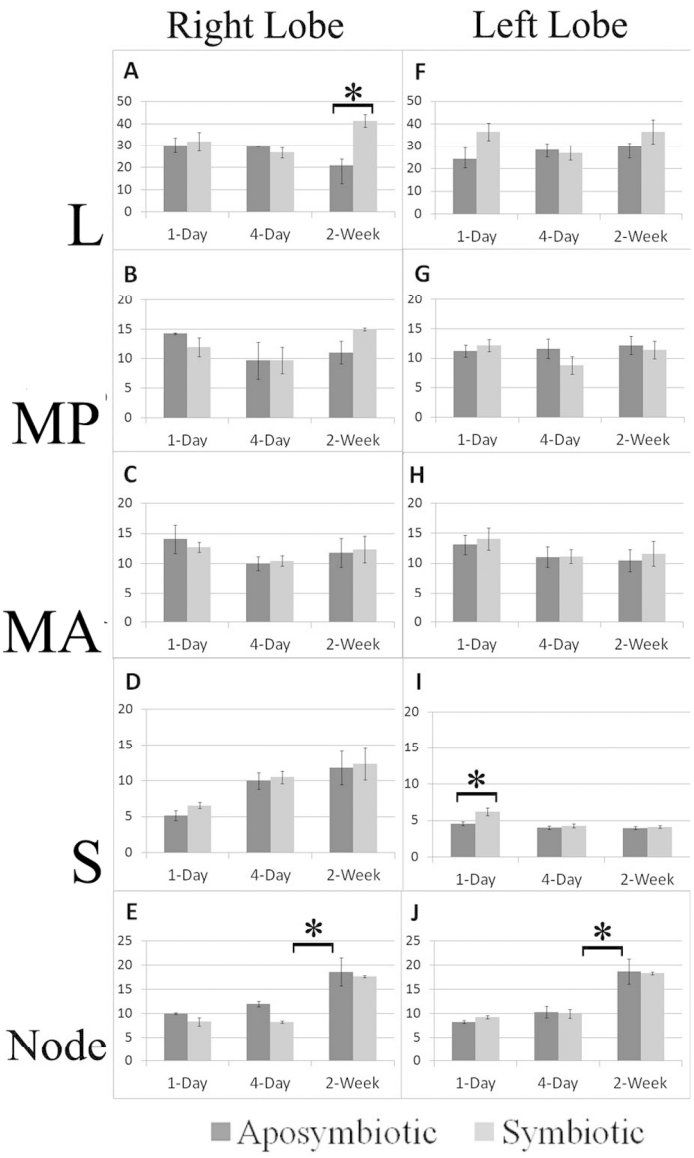


Figure 5. Vessels diameters in µm of light organ lobes comparing aposymbiotic and symbiotic animals in average ± standard error. Columns labeled at the top for the respective lobe. A and F. Largest vessel (L) diameters of 1-Day, 4-Day, and 2-week animals. B and G. MP diameters of 1-Day, 4-Day, and 2-week animals. C and H. MA diameters of 1-Day, 4-Day, and 2-week animals. D and I. Smallest vessel (S) diameters of 1-Day, 4-Day, and 2-week animals. E and J. Number of branch points (nodes) counted for each of the samples. Significance of P< 0.5 is indicated by asterisk (*).

170x291mm (600 x 600 DPI)