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THE EVOLUTIONARY ECOLOGY OF A SEPIOLID SQUID-VIBRIO ASSOCIATION: FROM CELL TO ENVIRONMENT

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VIBRIO
MUTUALISM
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ABSTRACT. – Mutualistic relationships between bacteria and their eukaryotic hosts have existed for millions of years, and such associations can be used to understand the evolution of these beneficial partnerships. The symbiosis between sepiolid squids (Cephalopoda: Sepiolidae), and their *Vibrio* bacteria (gamma Proteobacteria: Vibrionaceae), has been a model system for over 20 years, giving insight as to the specificity of the association, and whether the interactions themselves give rise to such finely tuned dialog. Since the association is environmentally transmitted, selection for specificity can evolve from a number of factors; abiotic (temperature, salinity), as well as biotic (host species, receptors, cell/cell interactions). Here, we examine the transition between these forces effecting the symbiosis, and pose possible explanations as to why this association offers many attributes for understanding the role of symbiotic competence.

INTRODUCTION

All metazoans form life-long beneficial partnerships with microbial symbionts. Most of these host-microbe interactions are highly specific and environmentally transferred, meaning that symbiont(s) must colonize the hosts each generation.

Bacterial associations that arise with these metazoan hosts have undergone substantial selective pressures that assure maintenance of specificity, as well as the ability of the microbial partner(s) to remain in the association without being detected by the host's immune capabilities (McFall-Ngai 2005). In order to establish and maintain such highly intimate associations, well-developed mechanisms must be in place to ensure and maintain successful colonization. These mechanisms often involve complex and well-regulated molecular signaling events that act as a type of "conversation" between the partners. Although the host and symbiont usually dictate a certain degree of selection through this molecular dialogue, particularly when mutualistic associations require that all partners participate in the relationship, it is the ecology that often determines whether symbionts that are environmentally transmitted possess a certain degree of flexibility each time they infect a new host (Colwell 1984). These life-history trade-offs between environment and host selection are equivalent in the manner in which they shape the evolution of these associations, as well as the interactions that are so important for maintaining stability within those populations (Anderson & May 1979).

A large number of mutualistic, marine associations have been vastly studied to examine the chasm between the ecology and cellular interactions that drive environmentally transmitted symbioses (Haddad *et al*. 1995, Haygood & Distel 1993, Hoegh-Guldberg *et al*. 2007, Newton *et al*. 2007). One type of symbiosis that has recently been developed as a model system includes the associations between sepiolid squids (Cephalopoda: Sepiolidae), and luminous bacteria (from the genera *Vibrio* and *Photobacterium*; McFall-Ngai & Ruby, 1991). Light organ symbioses occurring with luminous bacteria of the genera *Vibrio* and *Photobacterium* are found in two families of squids: Loliginidae and Sepiolidae (Fig. 1; Herring 1977, Mangold & Boletzky 1988). In most species of sepiolids, squids are bioluminescent, owing to the presence of bacterial symbionts contained in a complex, bilobed, light-emitting organ (Fig. 2; Montgomery & McFall-Ngai 1998, Nishiguchi *et al*. 2004). The light organ itself is used in a behavior termed counter-illumination or silhouette reduction (Jones & Nishiguchi 2004). Light produced by bacteria is used to match down-welling

Fig. 1 – The bobtail squid, *Euprymna scolopes*. Mantle length ~ 4 cm.

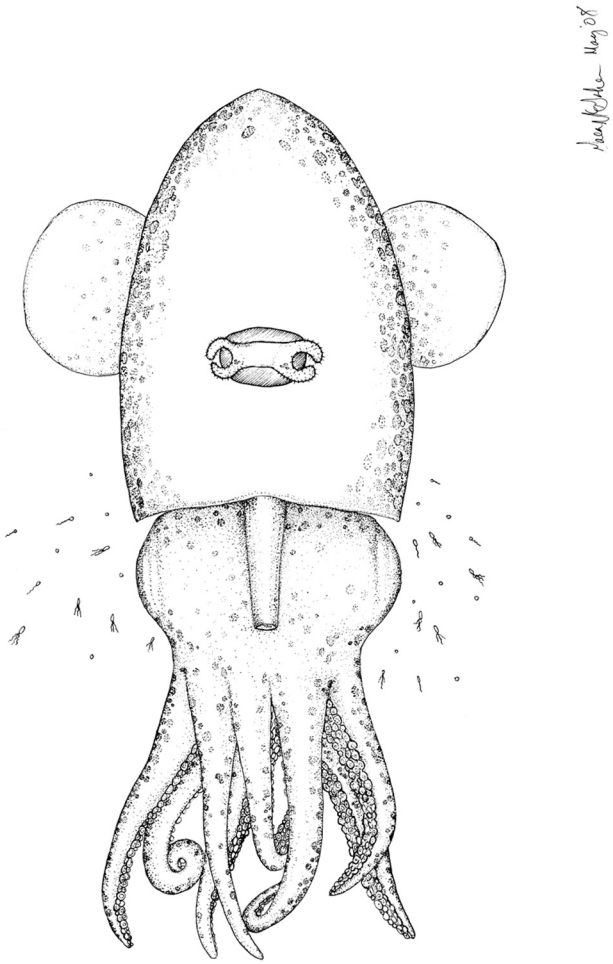

Fig. 2 – Cartoon diagram of *Euprymna*, with the placement of the light organ within the mantle cavity. During respiration/ventilation, the squid uptakes water laden with approximately 10^6 bacteria/mL. *Vibrio fischeri* bacteria comprise approximately < 1% of the total number of bacteria that are found in seawater surrounding host squids. Specificity may be influenced by changes in temperature, salinity, or competition between bacteria in the seawater prior to infection. Size of bacteria in reference to host squid is not to scale.

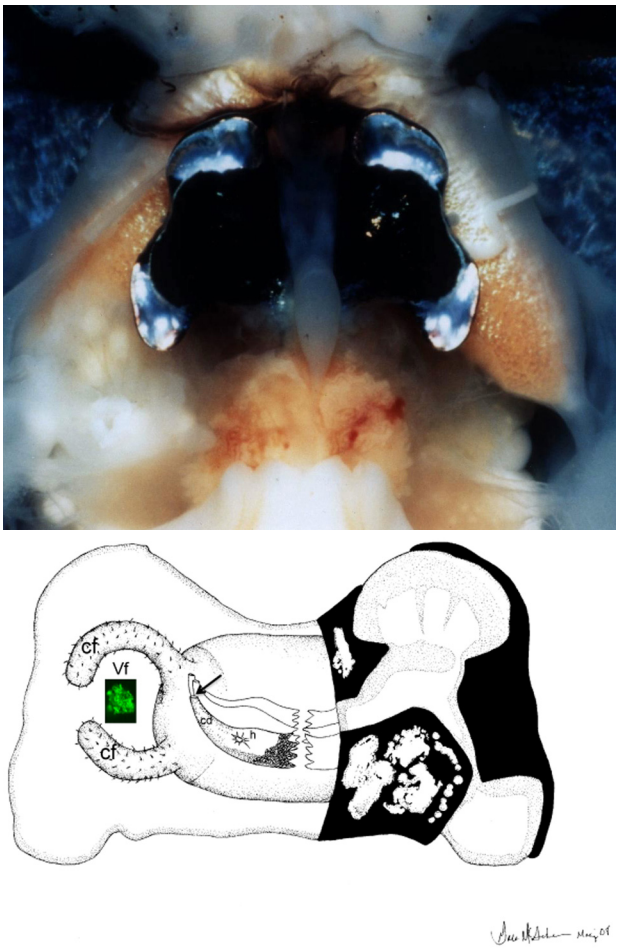

Fig. 3. – **A**, Central dissection of *E. scolopes*, exposing the light organ and ink sac surrounding the light organ complex. **B**, Schematic representation of the light organ, with one side exposed. After hatching, the ciliated fields (cf) create currents and secrete mucus that aggregate Gram-negative bacteria including cells of *Vibrio fischeri* (Vf, green) that out-compete non-symbiotic bacteria for space in these structures. Aggregated *V. fischeri* migrate through one of three pores (arrow) on either side of the light organ where they colonize the light organ after navigating past ciliated ducts (cd) and host hemocytes (h).

moonlight, so that squids avoid detection by predators or prey from below. The morphology of the light organ is such that all light is directed ventrally, which then diffuses through the mantle cavity and hides any shadow being produced (McFall-Ngai & Montgomery 1990). Emission of the bacterial luminescence is controlled in two ways: (i) by a host-modulated, diel (day/night fluctuations) restriction on the luminescent output per bacterial cell and, (ii) by a series of accessory tissues, which are functionally analogous to the tissues that modulate light quality in the eye (Boettcher *et al*. 1996).

One of these associations, that between the Hawaiian bobtail squid *Euprymna scolopes* and the bacterium *Vibrio fischeri*, has been extensively studied for the past 20 years, and has led to a number of rich discoveries for understanding the underlying mechanisms that regulate the interactions between bacteria and eukaryotic tissues (McFall-Ngai 2002). *E. scolopes* obtains bacteria from the environment each generation as a juvenile. The association is highly specific; *i.e*., only *V. fischeri* can colonize the tissues of a newly-hatched juvenile *E. scolopes* squid (McFall-Ngai & Ruby, 1991). The host squid houses its extracellular bioluminescent symbionts in a bilobed light organ that is part of the ink sac complex contained in the center of the squid's mantle cavity (Fig. 2, 3A). The light organ at this developmental stage is composed of a complex ciliated field with two sets of appendages that entrain seawater towards a set of six pores, three on each side of the light organ (Fig. 3B). Upon hatching, the host ventilats seawater containing a mixture of bacterial species through the mantle cavity. Free-living *V. fischeri* must then pass through these pores and migrate through ciliated ducts that terminate in three separate epithelia-lined crypt spaces on each side of the light organ (Fig. 4). It is in these

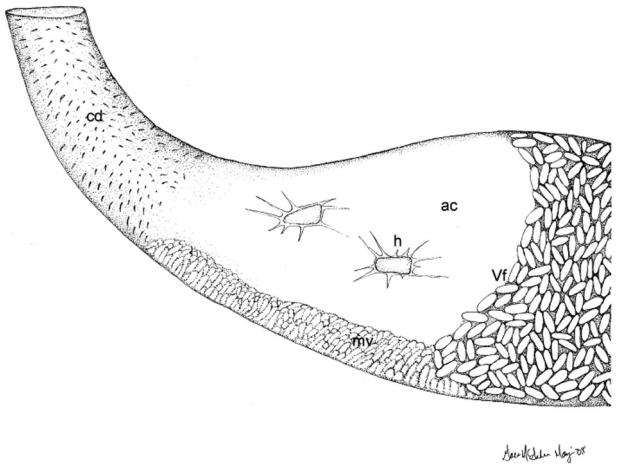

Fig. 4. – Diagram of one of the antechamber (ac) regions leading to the epithelial lined crypt spaces of the light organ. *V. fischeri* (Vf) must overcome a number of host-derived hurdles to successful colonization. Motility is required to traverse the ciliated duct (cd) where these cells also encounter potentially lethal host-derived reactive oxygen species. Successful *V. fischeri* cells swim through the ac and colonize microvilli (mv) of the epithelial cell surfaces of the light organ crypts. A number of cell-signaling events are involved with coordinating the molecular conversation needed for successful colonization (see text).

crypt spaces that colonization finally is established.

Over the past twenty years a number of studies have gone onto characterize a number of developmental effects that *V. fischeri* induces in the squid host, including programmed cell death or apoptosis, epithelial cell swelling, hemocyte trafficking, mucus shedding, and changes in host gene and protein expression (Chun *et al*. 2006, Doino Lemus & McFall-Ngai 2000, Foster & McFall-Ngai 1998, Kimbell & McFall-Ngai 2004, Koropatnick *et al*. 2004, Koropatnick *et al*. 2007, Lamarcq & McFall-Ngai 1998, McFall-Ngai & Ruby, 1991, Montgomery & McFall-Ngai 1994, Nyholm *et al*. 2002). It is the complexity of how *Vibrio* bacteria are first able to locate specific squid hosts in the aquatic environment, to sufficiently colonize this complex organ in a highly specific manner that pervades all levels of symbiosis: physiology, molecular specificity, immunology, and eventually speciation amongst different populations of *Vibrio* bacteria.

ABIOTIC FACTORS AND THE ENVIRONMENT

Vibrio bacteria are cosmopolitan species; they are commonly found in both fresh and oceanic waters including rivers and lakes, as well as a variety of marine habitats (coastal, pelagic deep sea) (Feldman & Buck 1984, Ramesh *et al*. 1989, Urakawa & Rivera 2006). They inhabit a number of ecological niches, such as within the natural bacterioplankton community (Colwell 1984), as saprophytes on dead or decaying matter (Andrews *et al*. 1984), as pathogens to both humans and other metazoans (Owens & Busico-Salcedo 2006, Wong & Wang 2004), and as commensals or mutualists with many marine invertebrates (Dunlap *et al*. 2007, Nyholm & McFall-Ngai 2004, Sawabe 2006). With such great breadth in their natural ability to adapt to such a wide diversity of habitats, it is of no surprise that variation exists among strains that are isolated from differing environments.

Abiotic factors have been previously shown to have substantial effects on the selection of highly adaptable Vibrio strains (Nealson & Hastings 1979). These include temperature, salinity, nutrient concentration, and UV irradiation (Czyz *et al*. 2000, Rosenberg *et al*. 2007, Ruby & Nealson 1978, Soto *et al*. 2008a, Soto *et al*. 2008b). For instance, temperature has significant effects on the shaping of ecological associations between *Vibrio* bacteria and their host organisms (Hilton *et al*. 2006, Larsen *et al*. 2004). Many virulence factors that influence colonization and infection are strongly regulated by temperature, and can have dramatic effects upon host populations, often killing large numbers of individuals (Rosenberg *et al*. 2007). For mutualistic Vibrios that colonize the light organs of sepiolid squids, there are multiple abiotic factors that may influence the ability of colonization to be effective, as well as which types of *Vibrio* species may be more prevalent in the light organ. Initial studies measuring the abundance and types of *V. fischeri* bacteria that were found in habitats containing *Euprymna scolopes* indicated that not only were symbiotically competent Vibrios found in Hawaiian waters, but many of those strains were not accountable by direct plating methods (Lee & Ruby 1995). Since sepiolid squid vent out 90-95% of their bacterial light organ composition each day with the onset of dawn (Boettcher *et al*. 1996), the bacteria released are viable and have a profound effect on the abundance of *V. fischeri* in habitats where host squids are abundant (Jones *et al*. 2007, Lee & Ruby 1994). This has been shown not only in Hawaiian *E. scolopes* populations (Lee & Ruby 1992), but in *E. tasmanica* populations throughout Australia (Jones *et al*. 2007). In addition, both morning and evening measurements infer that the diurnal venting behavior of *E. tasmanica* has an effect on the number of detectable bacteria in the water column during those times, particularly in Botany Bay, New South Wales, which is a semi- enclosed body of water with a constant population of *E. tasmanica*. The data suggests that interactions between *Vibrio* bacteria in the water column and resident host populations are very similar between the two *Euprymna* species.

In contrast, symbiotic *Vibrio* bacteria in Banyuls-sur-Mer, France, are more affected by temperature than by the behavior or specificity of resident host squids. *Sepiola*, which is the genus of sepiolids found in the Mediterranean, harbor two species of *Vibrio* bacteria, *V. fischeri* and *V. logei* (Fidopiastis *et al*. 1998). These two species of *Vibrio* are similar both genetically and in their phylogenetic placement among other Vibrios (Browne-Silva &

Nishiguchi 2008, Nishiguchi & Jones 2004, Nishiguchi & Nair 2003), but have subtle differences in their ecological niches. *V. logei* is a psychrophile, and is commonly found in colder temperatures compared to *V. fischeri*. Thus, when examining the population structure of *V. fischeri* and *V. logei* according to depth, the expected outcome was to observe differences in abundance between the two species. However, there was no difference in abundance of either species, despite previous results examining species of *Sepiola* collected at greater depths, which harbored more *V. logei* then *V. fischeri* (Nishiguchi 2000). What was evident from this study were differences between summer and winter collection periods of both *V. fischeri* and *V. logei*. Both species were significantly higher in their density in the winter sampling compared to the summer season, as well as the overall concentrations of bacteria in the Vibrionaceae. This may in part be due to the availability of nutrients during the winter when the thermocline disappears, as well as less competition for resources from other bacteria. Since both species are represented in higher concentrations during the winter than the summer months, this may also influence the amount of bacteria present in squids during those times of the year as well. Earlier studies suggest that no host specificity exists in either *V. fischeri* and *V. logei*, but rather temperature drives which *Vibrio* species is more prevalent in *Sepiola* (Nishiguchi 2000). Infection between these two species of *Vibrio* may be equally parsimonious, but may be temperature limited under high nutrient conditions (i.e., in squid light organs). Further studies examining the distribution of symbiotic *Vibrio* bacteria in areas where multiple species exist may help determine how strongly environment selects for specific features in competent bacteria, and whether factors such as temperature determines which symbiont is more capable of adapting and eventually evolving to a new ecological niche.

Salinity has also been a major abiotic factor for determining how well *Vibrio* bacteria are capable of infecting host tissues. All extant species of *Euprymna* are allopatric and found within the Indo-West Pacific, whereas species of *Sepiola* are found sympatrically in the Mediterranean Sea. Thus, *Vibrio* bacteria infecting *Euprymna* hosts are specialists, since they have a hierarchical degree to which they infect different *Euprymna* species (Nishiguchi *et al*. 1998). In contrast, *Sepiola* hosts share the same two species of *Vibrio*, and do not exhibit any competitive dominance during colonization (Nishiguchi 2000). Since there is a major difference as to how these two genera of sepiolid squids obtain their bacteria from the environment, there remains a number of mechanisms that influence how *Vibrio* bacteria respond to changes in salinity and temperature, both separately and synergistically. Previous evidence has already demonstrated how changes in osmolarity affect bioluminescence in symbiotic strains of *V. fischeri* ES114 (Stabb *et al*. 2004). Similarly, comparison of different *V. fischeri* strains from a number of *Euprymna* and *Sepiola* host species shows that *V. fischeri* strains from habitats with greater variation (*V. fischeri* ET101, from Melbourne, Australia), appear more sensitive to changes in both salinity and temperature than strains from more homogeneous environments (*V. fischeri* ES114, Kaneohe Bay, Hawaii; Soto *et al*. 2008a). The same is true for *V. fischeri* strains isolated from two closely related sympatric host species (*E. morsei* and *E. berryi*), but isolated from different habitats (Northern *vs*. Southern Japan). Both strains respond differently to salinity and temperature changes, and may be due to physiological differences resulting from evolution within their respective thermal niches. Interestingly, synergistic effects were also observed between strains grown under colder temperatures and lower salinities (12°C/24 ppt), where microbial allelopathy may also have an important role in determining competitive dominance (Soto *et al*. 2008a, Soto *et al*. 2008b). Therefore, competitive dominance in genera such as *Euprymna* may not be the sole result of native *Vibrio* symbionts having faster generation times than non-native ones upon colonization. Rather, *V. fischeri* strains may be competing prior to their infection while residing in the free-living bacterioplankton community, where the environment is much like minimal media relative to the nutrient rich light organ habitat.

ENVIRONMENTAL TRANSMISSION AND THE POPULATION ECOLOGY BETWEEN SEPIOLID SQUIDS AND *VIBRIO* BACTERIA

Environmentally transmitted symbionts are often subjected to broad and changing environmental regimes, where various factors select for adaptations that are suited for (1) host colonization and persistence, (2) the free-living pre-infective state, or (3) both ecological niches. Yet, broadly distributed host-symbiont populations, like those found in the sepiolid squid-*Vibrio* mutualism, may be subjected to different selective conditions that may result in different population structures due to adaptations occurring in the symbiont. Interestingly, while much work has been completed to identify cospeciating host/symbiont assemblages through studies of parallel cladogenesis (Nishiguchi 2002, Nishiguchi *et al*. 1998), few studies have examined how hosts or their environment may dictate symbiont genotypes, and whether symbionts are capable of host switching across large geographical distances through ecological adaptation.

With the exception of two genera, all sepiolid squids possess a light organ containing luminescent bacteria (Nesis 1982). Studies identifying symbiotic bacteria from different host squids indicate that the composition from each species of sepiolid is comprised of only one to three species of *Vibrio* (*V. fischeri*, *V. logei*, and *V. harveyi*) or *Photobacterium* (*P. leiognathi*), and that no other species of bacteria are found present inside the adult light organ

(Fidopiastis *et al*. 1998, Guerrero & Nishiguchi 2007, Nishiguchi *et al*. 2004). Since light organ pores are continually open to the surrounding seawater providing access for any type of bacterium into the light organ crypts (Fig. 2), the presence of only these species of *Vibrio* or *Photobacterium* in the light organ illustrates the specificity that prevents other types of bacteria from entering and colonizing the light organ (Visick & Ruby 2006).

Vibrio specificity is also hierarchical; all symbiotically competent vibrios are capable of colonizing sepiolid light organs, but native strain vibrios are better suited to colonize the crypts of their own squid host light organ when compared to non-native *Vibrio* competitors (Nishiguchi 2002, Nishiguchi *et al*. 1998). Because viable competent bacteria are "vented" every morning at dawn, this behavior selects for bacteria that have evolved specificity to a particular host, yet must still manage to survive in the surrounding environment once exuded from the light organ (Jones & Nishiguchi 2006, Nyholm & McFall-Ngai 2004).

Population studies examining both sepiolid squids and *Vibrio* bacteria have provided a roadmap for understanding the large-scale dynamics of how symbiotic bacteria are environmentally transmitted within and between host populations. Using haplotype networks in combination with nested clade analysis, variation among three species of *Euprymna* from the Indo-West Pacific (*E. scolopes*, Hawaii; *E. tasmanica*, Australia; and *E. hyllebergi*, Thailand) were examined to determine whether cospeciation was prevalent among all three species pairs (Jones *et al*. 2006). *Euprymna* species were genetically distinct from each other, with little or no migration over large geographical distances. In contrast, *Vibrio* populations contained a much more diverse number of haplotypes, suggesting that both host specificity as well as abiotic factors facilitating long-distance migration determines the population structure of the symbionts. This was especially prevalent between populations of *E. tasmanica* that were separated by temperature gradients (Maugean zone), with specific haplotypes affiliated with either colder (Melbourne and Tasmania) or warmer (Sydney, Great Barrier Reef) water populations.

Preliminary results from genetic studies of sympatric Mediterranean squid-*Vibrio* populations demonstrate separation among different host species as compared to their symbiont populations. Squid haplotypes were geographically localized, with little genetic variability among individuals of the same species from different populations (*i.e*., *S. affinis*). No shared haplotypes were found among different species of *Sepiola*. Conversely, *V. fischeri* and *V. logei* populations were found to be homogenzied in the same area, regardless of the squid host they were isolated from. Similar *Vibrio* haplotypes were found in hosts collected from both Banyuls-sur-Mer (S. France) and Bari, Italy (Adriatic Sea). In other words, a higher degree of genetic variation was found across the Western Mediterranean in terms of the symbiont population. This seems to point at the physical mobility and environmental range of the symbionts and not host specificity. These observations are in concordance with *Vibrio* populaion data from the Indo-west Pacific, where temperature, salinity, and currents either keep particular strains restricted (Maugean zone), or, if conditions are similar, provide inter-clonal exchange between large geographical distances (NE Australia, Hawaii, and Thailand with secondary recolonization events occurring in Hawaii (Jones *et al*. 2006)). *Vibrio* bacteria that are vented daily may be under direct selection by abiotic factors such as water movement, salinity, and temperature, which influences their distribution beyond host movement (Soto *et al*. 2008a, Soto *et al*. 2008b). Interestingly, temperature and salinity gradients between *Vibrio* populations such as those in the Mediterranean and the Adriatic Seas are relatively similar, which may provide a selective advantage for non-native vibrios invading environments where different host species reside at similar conditions. Therefore, if temperature, salinity, and currents keep particular strains restricted, can adaptation occur rapidly enough for *V. fischeri* symbionts to invade new host populations? If so, can temperature and salinity adaptation effect squid-host colonization in habitats where there may be mixing of multiple strains adapted to the same environmental regime? Are the genetic mechanisms for colonization similar enough to "leap frog" from one population to another to infiltrate new host populations, or does adaptation to warmer temperatures select for strains with a higher fitness at increased temperatures?

OVERCOMING CHALLENGES OF ENVIRONMENTAL TRANSMISSION

Horizontal transmission of symbionts often pose a problem for both partners in that both the host and symbiont must locate each other and successfully colonize and maintain the association each generation. In the *E. scolopes*/*V. fischeri* association the host and symbiont have evolved mechanisms for ensuring successful colonization very early in the relationship. Why are these mechanisms necessary in this association? Every half-second the juvenile squid ventilates about 1.3 ml of ambient seawater through its mantle cavity. Comprising less than 0.1% of the total ambient bacteria, *V. fischeri* occurs at fewer than 500 cells/ml in nature. Thus, on average no more than a single *V. fischeri* cell, occupying about one-millionth the volume of the mantle cavity, will be present during each ventilation. Without mechanisms to harvest them, the symbionts would have to find one of the six 15-μm pores on the light organ surface in less than one second before being expelled out of the mantle cavity and back into the environment. How do the partners overcome

these physical and environmental challenges?

In response to peptidoglycan (a major cell wall component of bacteria) the squid host begins to secrete mucus from the ciliated fields (Nyholm & McFall-Ngai 2004, Nyholm *et al*. 2000). *V. fischeri* along with a variety of other Gram-negative bacteria are able to aggregate in this host-derived mucus. Mucus secretions are extremely common in nature and a number of marine organisms use these secretions to harvest small particles from the water column, usually in feeding behaviors. However, in this association, the collection of bacteria from the environment is not one-sided nor is it a passive process. By some, as yet undescribed mechanism, *V. fischeri* is able to outcompete other environmental bacteria in the mucus biofilm and excludes these other cells in the aggregations before successfully colonizing the light organ (Nyholm & McFall-Ngai 2003). The mechanisms underlying this competitive advantage are not known, but *V. fischeri* is able to display positive chemotaxis towards sialic acid, a common component of the host mucus (DeLoney-Marino *et al*. 2003). Bacterial motility is also important during these initial stages of aggregation. Although non-motile mutants of *V. fischeri* can aggregate, they cannot go on to colonize the light organ (see below). Hyper-motile mutants are deficient in both their ability to form aggregations in the host mucus biofilms and thus do not colonize the light organ (Millikan & Ruby 2004).

RUNNING THE GAUNTLET

Once the symbionts aggregate in the host mucus they must then migrate through this mucus to the pores on the surface of the juvenile light organ (Figs. 3 & 4). When they breach this border, they face an assault from a number of host factors that help to ensure that only *V. fischeri* will colonize the light organ crypt spaces. First, these cells must traverse a long ciliated duct with very active host-derived currents that direct seawater out of the pores and into the mantle cavity. Once again, symbiont motility is critical in navigating this physically stressful environment. Non-motile mutants of *V. fischeri*, although capable of forming aggregations, the first step of the colonization process, never make it through the ciliated ducts and are thus excluded from the light organ.

Besides these physical barriers to colonization, the host, armed with an arsenal of reactive oxygen species (ROS), including a squid halide peroxidase and nitric oxide (NO), also presents a challenging chemical milieu to any entering bacterial cell (Davidson *et al*. 2004, Weis *et al*. 1996). In response to these assaults, the symbiont has evolved countermeasures to these host barriers. *V. fischeri* gets around this first challenge by possessing a periplasmic catalase that scavanges hydrogen peroxide, the critical substrate necessary for the production of hypohalous acid, the microbiocidal end product of the reaction catalyzed by the squid halide peroxidase (Visick & Ruby 1998). Nitric oxide and nitric oxide synthase (NOS) are both ROS species that can be associated with host innate immune responses and have been found in the ciliated fields, ducts, and crypt spaces of aposymbiotic (uncolonized) hatchling squid. After colonization both NO and NOS expression are reduced in the squid host, presumably in response to some as yet undescribed symbiont signal (Davidson *et al*. 2004, Visick & Ruby 1998).

Once *V. fischeri* crosses through the ducts they swim through a large epithelial-lined space referred to as the "antechamber" and reach the narrow epithelial crypt spaces where colonization and subsequent growth finally takes place (Sycuro *et al*. 2006). One additional hurdle that these potential symbionts encounter is a population of host macrophage-like hemocytes that appear to act as sentinels in this microenvironment (Nyholm & McFall-Ngai 1998).

Phagocytic hemocytes or macrophages are ubiquitous throughout the animal kingdom where they play an important role in innate defense against pathogens. Among invertebrates, which lack the acquired immune response associated with antibody production, phagocytic hemocytes often play a critical role in host defense (Kurata *et al*. 2006, Stuart & Ezekowitz 2008). In mollusks, phagocytic hemocytes are reported to be involved in defense against pathogens in many tissues and their associated lumina, and the ability of these cells to engulf potentially pathogenic bacteria has been reported (Bayne *et al*. 2001, Canesi *et al*. 2002). However, the role these cells may play in interacting with beneficial bacteria is poorly understood. *E. scolopes* hemocytes traverse the epithelium into the crypt spaces where the symbionts reside and appear to 'sample' these spaces, not unlike the way that mammalian blood cells sample enteric microbiota (Fig. 4). Within the crypts of newly colonized juvenile *E. scolopes*, hemocytes have been observed with internalized bacterial cells (Nyholm & McFall-Ngai 1998). However, within the crypts of adult squid, hemocytes have never been observed to have engulfed bacteria although they are entirely surrounded by *V. fischeri* cells. These preliminary observations suggest that the squid's hemocytes change in response to the persistent presence of the symbionts, perhaps as part of the complex developmental program induced in the host by *V. fischeri*. The role that these hemocytes play in the light organ crypt spaces is at present unknown. They may be there as part of the regulatory function of the host used to maintain the symbiont population, or perhaps they prevent non-symbiotic interlopers from taking hold and out-competing the native symbiont population. Current studies in this system are focusing on how these immune cells interact with symbiotic and non-symbiotic environmental bacteria common to the host's natural environment and how these responses may change during development.

MOLECULAR BASIS OF THE CONVERSATION

Specificity, at least in the *E. scolopes*/*V. fischeri* symbiosis, appears to be established by a number of events, each of which appears to be well regulated and mediated through a fine-tuned molecular conversation that occurs between the symbiont and host. A number of genomics tools have recently become available to researchers that study this association, including a completed and annotated genome for *V. fischeri* and a cDNA expression library for colonized and uncolonized host light organs at various time points during the first three days of the symbiosis (Chun *et al*. 2006, Ruby *et al*. 2005). These new tools will serve as a roadmap for teasing apart and interpreting the host and symbiont genes involved with regulating this conversation. A number of experimental studies have also added insight into how some aspects of this dialogue work. For example, many of the symbiont-induced developmental phenotypes that have been observed in the host appear to be caused by microbial-associated molecular patterns (MAMPs) associated with the symbiont. *V. fischeri* lipopolysaccharide (LPS), peptidoglycan, and a derivative of peptidoglycan called tracheal cytotoxin (TCT) have been shown to induce host apoptosis, mucus secretion and cell regression respectively in *E. scolopes* (Foster *et al*. 2000, Koropatnick *et al*. 2004, Nyholm *et al*. 2002).

The host, in turn must have receptors and well-regulated signaling cascades to recognize and interpret these symbiont cues. MAMPs, especially those constituents that contribute to the cell walls and outer membranes of bacteria are known to interact with a variety of these animal and plant cell receptors (Bittel & Robatzek 2007, Nurnberger *et al*. 2004). A family of peptidoglycan recognition proteins (PGRPs) is known to exist in diverse animal phyla from flies to humans (Chaput & Boneca 2007). Recently, four homologues of the PGRP family have been described in *E. scolopes* from sequencing of an expressed sequence tag (EST) gene expression library (Chun *et al*. 2006, Goodson *et al*. 2005). It is presently unknown what role these receptors may play in the association, but ongoing research is being conducted to determine when and where these PGRPs are expressed during development and if their expression changes in response to the symbiont (M McFall-Ngai, pers comm). MAMPs are also known to stimulate host signaling and gene expression via the Toll/NFkB signaling pathway, another evolutionarily conserved signaling pathway for which *E. scolopes* has several homologues, although like the PGRPs, the function has yet to be determined (Goodson *et al*. 2005).

Many of these signaling pathways are directly tied to effector mechanisms of immune responses. Increasing evidence suggests that in both invertebrates and vertebrates, the host innate immune system plays a critical role in mediating communication between hosts and microbes and these "communications" maybe far more complex than previously thought (Dethlefsen *et al*. 2007). The "simplicity" of invertebrate models has proven valuable in our understanding of the function of these highly conserved molecular interactions. However, the squid/bioluminescent bacterial associations offer the broader research community an opportunity to go a step further and explore how specificity between animal hosts and symbionts can evolve and become fine-tuned to distinguish between a wide range of closely-related hosts and symbionts. Previous work has demonstrated a hierarchy to colonization between different strains of *V. fischeri* and their squid hosts (Nishiguchi *et al*. 1998). Such highly evolved specificity likely involves, not only adaptation to the physical environmental niche of each association but, also selection for highly specific receptor/ligand interactions.

SUMMARY

Interactions between hosts, symbionts, and the environment are becoming more complex as we progress to a better understanding of the molecular dialog between the partners. Environmentally transmitted symbiosis in particular, are set in a natural tug-of-war between balancing selective pressures from both the environment and the host that houses the symbiosis. Sepiolid squids and their *Vibrio* bacteria in general have a number of strategies that ensure that the association is successful (specificity), as well as allowing enough flexibility to allow adaptation to occur between various populations of host squids. This alternative strategy of accommodating a number of ecologically adaptable *Vibrio* strains not only allows for squid hosts to select for the best-fit symbionts, but also permits the bacteria to rapidly change in response to both the host and the environment in which the association is found (Gillespie & Turelli 1989, Leroi *et al*. 1994). Understanding how bacteria are capable of adapting to fluctuating environments has many implications that can be extrapolated to how organisms evolve both during and after transitions between habitats. Indeed, factors such as phenotypic plasticity, genetic polymorphisms, and a wide ecological breadth will influence how an organism responds to a new environment, and whether the phenotypic or genotypic response to the environment or host improves the fitness of the individual after those changes have occurred (Parmesan *et al*. 2005, Schlichting & Pigliucci 1993). Clearly, there is a need to understand how quickly organisms can adapt to different environments; with the noticeable change in global climate, it is to our benefit to determine if these changes will increase or decrease biodiversity on a much larger scale. This process is one of the major factors that will determine whether an organism will succeed or eventually go extinct in our world today.

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REFERENCES

Anderson RM, May RM 1979. Population biology of infectious diseases: part 1. *Nature* 280: 361-367.

Andrews CC, Karl DM, Small LF, Fowler SW 1984. Metabolic activity and bioluminescence of oceanic faecal pellets and sediment trap particles. *Nature* 307: 539-541.

Bayne CJ, Hahn UK, Bender RC 2001. Mechanisms of molluscan host resistance and of parasite strategies for survival. *Parasitology* 123 Suppl: S159-167.

Bittel P, Robatzek S 2007. Microbe-associated molecular patterns (MAMPs) probe plant immunity. *Curr Opin Plant Biol* 10: 335-341.

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 B* 179: 65-73.

Browne-Silva J, Nishiguchi MK 2008. Gene sequence of the *pil* operon reveal relationships between symbiotic strains of *Vibrio fischeri*. *Int J Syst Evol MIcrobiol* 58: 1292-1299.

Canesi L, Gallo G, Gavioli M, Pruzzo C 2002. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. *Microsc Res Tech* 57: 469-476.

Chaput C, Boneca IG 2007. Peptidoglycan detection by mammals and flies. *Microbes Infect* 9: 637-647.

Chun CK, Scheetz TE, Bonaldo Mde F, Brown B, Clemens A, Crookes-Goodson W J, Crouch K, DeMartini T, Eyestone M, Goodson MS, Janssens B, Kimbell JL, Koropatnick TA, Kucaba T, Smith C, Stewart JJ, Tong D, Troll JV, Webster S, Winhall-Rice J, Yap C, Casavant TL, McFall-Ngai MJ, Soares MB 2006. An annotated cDNA library of juvenile *Euprymna scolopes* with and without colonization by the symbiont *Vibrio fischeri*. *BMC Genomics* 7: 154.

Colwell RR 1984. Vibrios in the environment. John Wiley and Sons, New York.

Czyz A, Wrobel B, Wegrzyn G 2000. *Vibrio harveyi* bioluminescence plays a role in stimulation of DNA repair. *Microbiol* 146: 283-288.

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.

DeLoney-Marino CR, Wolfe AJ, Visick KL 2003. Chemoattraction of *Vibrio fischeri* to serine, nucleosides, and N-actylneuraminic acid, a component of squid light-organ mucus. *Appl Environ Microbiol* 69: 7527-7530.

Dethlefsen L, McFall-Ngai M, Relman DA 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 449: 811-818.

Doino Lemus J, McFall-Ngai MJ 2000. Alterations in the proteome of the *Euprymna scolopes* light organ in response to symbiotic *Vibrio fischeri*. *Appl Environ Microbiol* 66: 4091-4097.

Dunlap PV, Ast JC, Kimura S, Fukui A, Yoshino T, Endo H 2007. Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. *Cladistics* 23: 507-532.

Feldman KA, Buck JD 1984. Distribution and characterization of luminescent bacteria in a temperate estuary. *Estuaries* 7: 93-97.

Fidopiastis P M, Boletzky Sv, Ruby EG 1998. A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. *J Bacteriol* 180: 59-64.

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.

Foster JS, McFall-Ngai MJ 1998. Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelial tissues. *Dev Genes Evol* 208: 295-303.

Gillespie JH, Turelli M 1989. Genotype-environment interaction and the maintenance of polygenic variation. *Genetics* 121: 129-138.

Goodson MS, Kojadinovic M, Troll JV, Scheetz TE, Casavant TL, Soares MB, McFall-Ngai MJ 2005. Identifying components of the NF-kappaB pathway in the beneficial *Euprymna scolopes-Vibrio fischeri* light organ symbiosis. *Appl Environ Microbiol* 71: 6934-6946.

Guerrero RC, Nishiguchi MK 2007. Identification of light organ symbionts from the genera *Uroteuthis*, *Loliolus*, and *Euprymna* (Mollusca: Cephalopoda). *Cladistics* 23: 1-10.

Haddad A, Camacho F, Durand P, Cary SC 1995. Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete *Alvinella pompejana*. *Appl Environ Microbiol* 61: 1679-1687.

Haygood MG, Distel DL 1993. Bioluminescent symbionts of flashlight fishes and deep-sea anglerfishes from unique lineages related to *Vibrios*. *Nature* 363: 154-156.

Herring PJ 1977. Luminescence in Cephalopods and Fish. *Symp Zool Soc Lond* 38: 127-159.

Hilton T, Rosche T, Froelich B, Smith B, Oliver J 2006. Capsular polysaccharide phase variation in *Vibrio vulnificus*. *Appl Environ Microbiol* 72: 6986-6993.

Hoegh-Guldberg O, Muller-Parker G, Cook CB, Gates RD, Gladfelter E, Trench RK, Weis VM 2007. Len Muscatine (1932-2007) and his contributions to the understanding of algal-invertebrate endosymbiosis. *Coral Reefs* 26: 731-739.

Jones BW, Lopez JL, Huttenberg J, Nishiguchi MK 2006. Population structure between environmentally transmitted Vibrios and bobtail squids using nested clade analysis. *Mol Ecol* 15: 4317-4329.

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

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

Jones BW, Nishiguchi MK 2006. *Vibrio fischeri* transcripts reveal adaptations in an environmentally transmitted symbiosis. *Can J Microbiol* 52: 1218-1227.

Kimbell JR, McFall-Ngai MJ 2004. Symbiont-induced changes in host actin during the onset of a beneficial animal-bacterial association. *Appl Environ Microbiol* 70: 1434-1441.

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.

Koropatnick TA, Kimbell JR, McFall-Ngai MJ 2007. Responses of host hemocytes during the initiation of the squid-*Vibrio* symbiosis. *Biol Bull* 212: 29-39.

Kurata S, Ariki S, Kawabata S 2006. Recognition of pathogens and activation of immune responses in *Drosophila* and horseshoe crab innate immunity. *Immunobiology* 211: 237-249.

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.

Larsen MH, Blackburn N, Larsen JL, Olsen JE 2004. Influences of temperature, salinity and starvation on the motility and chemotactic response of *Vibrio anguillarum*. *Microbiology* 150: 1283-1290.

Lee KH, Ruby EG 1992. Detection of the light organ symbiont, *Vibrio fischeri* in Hawaiian seawater using *lux* gene probes. *Appl Environ Microbiol* 58: 942-947.

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.

Lee KH, Ruby EG 1995. Symbiotic role of the viable but nonculturable state of *Vibrio fischeri* in Hawaiian coastal seawater. *Appl Environ Microbiol* 61: 278-283.

Leroi AM, Lenski RE, Bennett AF 1994. Evolutionary adaptation to temperature. III. Adaptation of *Escherichia coli* to a temporally varying environment. *Evolution* 48: 1222-1229.

Mangold K, Boletzky Sv 1988. Mediterranean cephalopod fauna. I*n* The Mollusca, MR Clarke & Trueman ER eds. Academic Press, San Diego, Calif: 315-330

McFall-Ngai MJ 2002. Unseen forces: the influence of bacteria on animal development. *Dev Biol* 242: 1-14.

McFall-Ngai MJ 2005. The interface of microbiology and immunology: A comparative analysis of the animal kingdom. *In* The influence of cooperative bacteria on animal host biology, MJ McFall-Ngai, B Henderson & EG Ruby eds Cambridge University Press, New York, NY: 35-56

McFall-Ngai MJ, 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 MJ, Ruby EG 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* 254: 1491-1494.

Millikan DS, Ruby EG 2004. *Vibrio fischeri* flagellin A is essential for normal motility and for symbiotic competence during initial squid light organ colonization. *J Bacteriol* 186: 4315-4325.

Montgomery MK, McFall-Ngai M 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna 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.

Nealson KH, Hastings JW 1979. Bacterial bioluminescence: its control and ecological significance. *Microbiol Rev* 43: 496-518.

Nesis KN 1982. Cephalopods of the world. TFH Publications, Neptune City, NJ.

Newton ILG, Woyke T, Auchtung TA, Dilly GF, Dutton RJ, Fisher MC, Fontanez KM, Lau E, Stewart FJ, Richardson PM, Barry KW, Saunders E, Detter JC, Wu D, Eisen JA, Cavanaugh CM 2007. The *Calyptogena magnifica* chemoautotrophic symbiont genome. *Science* 315: 998-1000.

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

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

Nishiguchi MK, Jones BW 2004. Microbial biodiversity within the Vibrionaceae. *In* Origins, evolution, and the biodiversity of microbial life, J Seckback ed Cole-Kluwer Academic Publishers, Dordrecht, The Netherlands: 531-548.

Nishiguchi MK, Lopez JE, Boletzky Sv 2004. Enlightenment of old ideas from new investigations: The evolution of bacteriogenic light organs in squids. *Evol Dev* 6: 41-49.

Nishiguchi MK, Nair VA 2003. Evolution of pathogenicity and symbioses in Vibrionaceae: An combined approach using molecules and physiology. *Int J Syst Bacteriol* 53: 2019-2026.

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: 3209-3213.

Nurnberger T, Brunner F, Kemmerling B, Piater L 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev* 198: 249-266.

Nyholm SV, Deplancke B, Gaskins HR, Apicella MA, McFall-Ngai MJ 2002. Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl Environ Microbiol* 68: 5113-5122.

Nyholm SV, McFall-Ngai MJ 1998. Sampling the light-organ microenvironment of *Euprymna scolopes*: description of a population of host cells in association with the bacterial symbiont *Vibrio fischeri*. *Biol Bull* 195: 89-97.

Nyholm SV, McFall-Ngai MJ 2003. Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. *Appl Environ Microbiol* 69: 3932-3937.

Nyholm SV, McFall-Ngai MJ 2004. The winnowing: establishing the squid-vibrio symbiosis. *Nat Rev Microbiol* 2: 632-642.

Nyholm SV, Stabb EV, Ruby EG, McFall-Ngai MJ 2000. Establishment of an animal-bacterial association: recruiting symbiotic vibrios from the environment. *Proc Natl Acad Sci USA* 97: 10231-10235.

Owens L, Busico-Salcedo N 2006. *Vibrio harveyi*: pretty problems in paradise. *In* The Biology of the Vibrios, BAJSFL Thompson, ed ASM Press, Washington, DC: 266-280

Parmesan C, Gaines S, Gonzalez L, Kaufman DM, Kingsolver J, Townsend Peterson A, Sagarin R 2005. Empirical perspectives on species borders: from traditional biogeography to global climate change. *Oikos* 108: 58-75.

Ramesh A, Loganathan BG, Venugopalan VK 1989. Seasonal distribution of luminous bacteria in the sediments of a tropical estuary. *J Gen Appl MIcrobiol* 35: 363-368.

Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I 2007. The role of microorganisms in coral health, disease and evolution. *Nature Reviews Microbiology* 5: 355-362.

Ruby EG, Nealson KH 1978. Seasonal changes in the species composition of luminous bacteria in nearshore seawater. *Limnol Oceangr* 23: 530-533.

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, Whistler K Visick C, Greenberg EP 2005. Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proc Natl Acad Sci USA* 102: 3004-3009.

Sawabe T 2006. The mutual partnership between *Vibrio halioticoli* and abalones. *In* The Biology of the Vibrios, FL Thompson, B Austin & J Swings eds ASM Press, Washington, DC: 219-230.

Schlichting CD., Pigliucci M 1993. Control of phenotypic plasticity via regulatory genes. *Am Nat* 142:366-370.

Soto W, Gutierrez J, Remmenga MD, Nishiguchi MK 2008a. Synergistic effects of temperature and salinity on *Vibrio* symbionts from the Indo-west Pacific. *Microbiol Ecol* DOI 10.1007/s00248-008-9412-9.

Soto W, Lostroh CP, Nishiguchi MK 2008b. Physiological responses to stress in the Vibrionaceae: Aquatic microorganisms frequently affiliated with hosts. *In* Cooperation and stress in biology, J Seckback & Grube M eds Springer, New York, NY: in press.

Stabb EV, Butler MS, Adin DM 2004. Correlation between osmolarity and luminescence of symbiotic *Vibrio fischeri* strain ES114. *J Bacteriol* 186: 2906-2908.

Stuart LM, Ezekowitz RA 2008. Phagocytosis and comparative innate immunity: learning on the fly. *Nat Rev Immunol* 8: 131-141.

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.

Urakawa H, Rivera ING 2006. Aquatic environment. *In* The Biology of the Vibrios FL Thompson, B Austin, J Swings eds ASM Press, Washington, DC: 175-189.

Visick KL, Ruby EG 1998. The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. *J Bacteriol* 180: 2087-2092.

Visick KL, Ruby EG 2006. *Vibrio fischeri* and its host: it takes two to tango. *Curr Opin Microbiol* 9: 632-638.

Weis VM, Small AL, McFall-Ngai MJ 1996. A peroxidase related to the mammalian antimicrobial protein myeloperoxidase in the *Euprymna-Vibrio* mutualism. *Proc Natl Acad Sci USA* 93: 13683-13688.

Wong HC, Wang P 2004. Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *J Applied Microbiol* 96: 359-366.

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