Advances in Environmental Microbiology 2

Christon J. Hurst *Editor*

The Mechanistic Benefits of Microbial Symbionts

Springer

Advances in Environmental Microbiology

Volume 2

Series editor
Christon J. Hurst
Cincinnati, Ohio
USA

and

Universidad del Valle
Santiago de Cali, Valle
Colombia

Hawaiian bobtail squid, *Euprymna scolopes*. Courtesy of Margaret McFall-Ngai

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Christon J. Hurst
Editor

The Mechanistic Benefits of Microbial Symbionts

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Chapter 11
Fiat Lux: The Squid–Vibrio Association as a Model for Understanding Host–Microbe Associations

Spencer V. Nyholm

Abstract The symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* offers an experimentally tractable model for understanding the role of beneficial bacteria on animal development and the mechanisms by which host and symbionts establish and maintain highly specific associations. The symbiont is transmitted from the environment each generation, and mechanisms must be in place to ensure specificity. Research over the years has revealed some of the "molecular dialogue" that occurs between the partners during and after colonization. Many of these interactions involve microbe-associated molecular patterns (MAMPs) and host pattern recognition receptors (PRRs) as well as components of the host's innate immune system. The role of light production by the symbiont and light detection by the host is also critical to the association and has likely served as a driving force during the evolution of this symbiosis. Finally, the host harbors a second symbiosis, housing a consortium of bacteria in the female reproductive system. *Euprymna scolopes* therefore offers the unique opportunity to study both a binary and consortial symbiosis in the same host.

11.1 Importance of Model Associations in Symbiosis Research

All animals and plants form beneficial associations with microorganisms, and such associations have had a profound effect on the evolution of these groups (McFall-Ngai et al. 2013; Oldroyd 2013). In recent years it has become evident that symbionts play a critical role in the development and health of not only individual hosts, but entire ecosystems [e.g., coral reefs; see Chap. 10 by V. Weis and hydrothermal vent and other chemoautotrophic ecosystems (Dubilier et al.

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C.J. Hurst (ed.), *The Mechanistic Benefits of Microbial Symbionts*, Advances in Environmental Microbiology 2, DOI 10.1007/978-3-319-28068-4_11

2008)]. Understanding the molecular mechanisms by which these associations are established and maintained can be difficult because often the symbionts occur as complex consortia where delineating the role of any one member is challenging. Therefore, employing the use of model systems with fewer partners is often advantageous. The binary association between the Hawaiian bobtail squid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* is one such system that has been used to understand how beneficial host–microbe associations are formed (McFall-Ngai 2014). This chapter will review how this highly specific association is established and maintained, highlighting the molecular mechanisms by which the partners communicate to achieve this goal and how the host's innate immune system contributes to the specificity of the symbiosis.

11.2 Bioluminescent Symbioses

Bioluminescence (the production of light by living organisms) is a common biological phenomenon in many environments but is especially common in marine ecosystems (Widder 2010). The light produced by organisms is used for a number of behaviors including finding prey, camouflage to avoid predation, and attracting mates. The vast majority of fauna use autogenic bioluminescence, meaning they produce the chemicals (substrate luciferin and enzyme luciferase) necessary for light generation. A few groups, mainly found among fishes and squid, rely on a symbiotic relationship with bacteria for light production.

The majority of bioluminescent bacteria in the marine environment belong to members of the *Gammaproteobacteria* group *Vibrionaceae* and primarily within the genera *Vibrio* and *Photobacterium* (Guerrero-Ferreira et al. 2013; Urbanczyk et al. 2011). Members of these groups have formed associations with a number of different species of fish and squid (Guerrero-Ferreira and Nishiguchi 2007; Urbanczyk et al. 2011). Perhaps the best studied of this group is *Vibrio fischeri* (also referred to as *Aliivibrio fischeri*) which produces light through a process known as quorum sensing. This phenomenon, which was first discovered in *V. fischeri*, regulates light production based on density-dependent cell–cell communication [reviewed in Verma and Miyashiro (2013) and Miyashiro and Ruby (2012)]. *Vibrio fischeri* produces a freely diffusible chemical autoinducer known as an N-acyl homoserine lactone (3-oxo-C6-HSL) that initiates gene expression when a quorum or critical density of bacterial cells is present (e.g., as found in culture or contained within the light organ of a host). The chemistry of bacterial bioluminescence in *V. fischeri* is based on production of an enzyme (luciferase) that oxidizes substrates [bacterial luciferin; reduced flavin mononucleotide (FMNH2)] and a long-chain fatty acid (RCHO) into FMN and aliphatic acid (RCOOH). The genes for all of these factors are encoded by the *lux* operon (Lux ICDABEG) (Gray and Greenberg 1992) and are transcriptionally activated when 3-oxo-C6-HSL binds the LuxR activator. A positive feedback loop allows for the production of more autoinducer (LuxI) and thus increases luminescence output. Luminescence in *V. fischeri* is also regulated by

two other quorum sensing systems AinS–AinR and LuxS–LuxP/Q [reviewed in Verma and Miyashiro (2013)]. Unlike many nutritional symbioses discussed in other chapters of this book, luminescence (light production) is the main selective force in bioluminescent symbioses.

11.3 The Association Between *Euprymna scolopes* and *Vibrio fischeri*

The association between the model cephalopod *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* has been used for over 25 years now to understand interactions of animal hosts with beneficial bacteria (McFall-Ngai 2014). This association has many advantages that lend themselves to the study of host–microbe interactions. For example, each partner can be raised independently in the laboratory and is readily available for molecular, biochemical, and genetic analyses. The female host lays clutches of hundreds of eggs that hatch after an approximate 20-day embryogenesis, allowing a high sample number of squid for any given experiment. Numerous researchers are currently maintaining squid rearing facilities, and a small cohort of animals (typically 10–20 breeding pairs) will yield approximately 20,000–60,000 juvenile squid per year that may be used for experimentation. The establishment of the symbiosis takes place over a short time frame, i.e., colonization occurs within hours after the host hatches from egg cases and therefore experiments can often quickly be resolved.

Euprymna scolopes is a relatively small squid (average adult length = 30–40-mm) that is an active nocturnal predator endemic to the Hawaiian archipelago. It belongs to a family of squid known as the Sepiolidae whose members are found in the Indo-Pacific and Mediterranean Sea and often form associations with bioluminescent bacteria. As with other bioluminescent hosts, a defining feature of *E. scolopes* is the presence of a bilobed light organ that is located in the center of the mantle cavity and is part of the hindgut-ink sac complex (McFall-Ngai and Montgomery 1990). The light organ itself is made up of a number of complex tissues including a lens, reflective tissue, and epithelium-lined crypt spaces that house the extracellular symbionts (to densities of 10^9 *V. fischeri* cells per adult squid). The crypt spaces are connected to the environment via a ciliated duct that terminates at a pore on either side of the light organ (Fig. 11.1). Like other squid, *E. scolopes* is relatively short lived (9 months to 1 year). Juvenile squid hatch from externally laid egg cases that are deposited in the environment among coral reefs and shallow sand flats. Embryogenesis is approximately 3 weeks, and juvenile squid hatch without their symbionts and thus must be colonized by *V. fischeri* from the environment each generation. *Euprymna scolopes* is a nocturnal predator that hunts for small crustaceans near coral reefs. The light produced by the bacteria is used to camouflage the host in a behavior known as counterillumination (Jones and Nishiguchi 2004). The host is able to match down-welling moonlight and

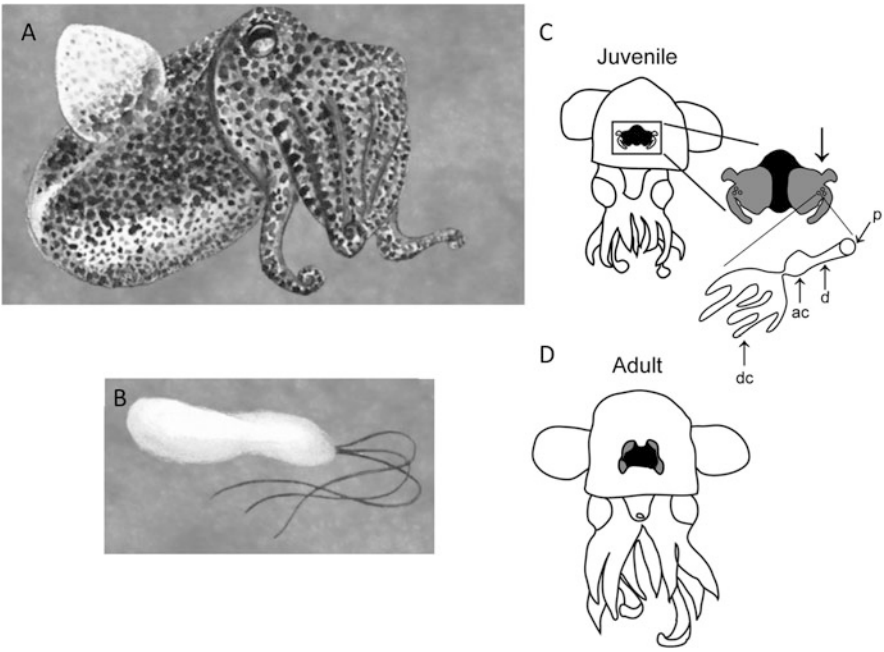

Fig. 11.1 The *Euprymna scolopes–Vibrio fischeri* symbiosis. The Hawaiian bobtail squid (**a**) forms a highly specific association with the bioluminescent bacterium *Vibrio fischeri*. (**b**) The *V. fischeri* bacteria are acquired from the environment and colonize the nascent host light organ for each new generation of squid. *Vibrio fischeri* is flagellated and its motility is crucial during the early stages of colonization. (**c**) Ventral surface of a juvenile squid. The nascent light organ is located in the center of the mantle cavity and includes superficial ciliated epithelial fields (*gray*). The interior of one juvenile light organ crypt system, including the pores (p), ducts (d), antechambers (ac), and the deep crypts (dc). (**d**) Ventral surface of an adult squid showing the mature light organ (**a** and **b** drawn by Andrea Suria; **c** and **d** are adapted from Rader and Nyholm (2012))

starlight such that the silhouette that would normally be viewed by predators is obscured. In exchange, the symbionts are housed in the light organ where they receive all of their nutrition from the host.

Vibrio fischeri is a cosmopolitan bacterium that is found throughout the world's oceans. In Kaneohe Bay on the island of Oahu, Hawaii, *V. fischeri* populations are as much as 24–30 times higher in areas that also harbor the Hawaiian bobtail squid compared to surrounding waters that lack *E. scolopes* (Lee and Ruby 1994; Jones et al. 2007). This increase in the abundance of bacteria in the squid's habitat is likely due to a unique diel rhythm that occurs in this association (Fig. 11.2). While the host is hunting at night, it has a full complement of *V. fischeri* such that the symbionts are at a high cell density and bioluminescence is induced (see above). At dawn, the host undergoes a quiescent period where it buries in the substrate. At this time and in response to a light stimulus (sunlight), the musculature of the light organ contracts and expels the contents of the crypt spaces into the surrounding seawater (Boettcher et al. 1996; Nyholm and McFall-Ngai 1998). Approximately 95 % of the symbionts are expelled, and the remaining 5 % of *V. fischeri* cells divide

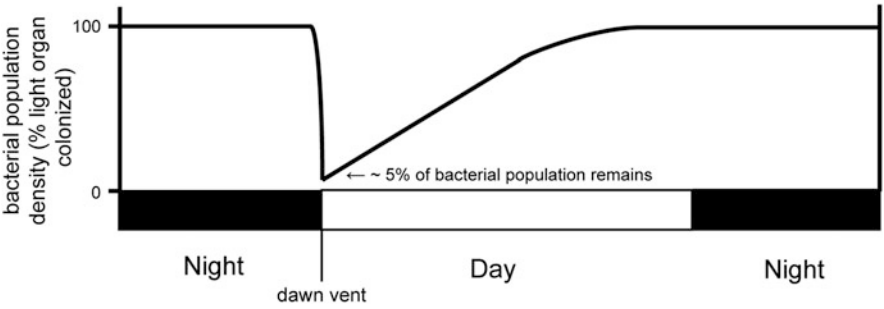

Fig. 11.2 Host-symbiont daily rhythm. The squid–vibrio association undergoes a diel rhythm whereby the light organ contains a full symbiont complement when the host is hunting at night. Because *V. fischeri* cells are at a high density during this time, the genes for bioluminescence are activated, and light is produced which the host in turn uses as a means of camouflage known as counterillumination. At dawn each day, 95 % of the symbionts are expelled, and the remaining symbionts divide during the day to repopulate the organ. This rhythm serves to regulate the symbiont population and to seed the environment with viable *V. fischeri* [Adapted from Rader and Nyholm (2012)]

during the day so that there is once again a full population when the host emerges at dusk to hunt. The squid's light organ can therefore be thought of as a chemostat whereby this "bioreactor" leads to a daily growth and expulsion of large numbers of *V. fischeri*. The expelled bacteria are viable and able to transition to a free-living state. These active and motile cells are then capable of colonizing the next generation of squid. Dawn therefore represents a dramatic transition in the association. Not only are the symbionts expelled, but the exudate also contains shed apical surfaces of host epithelial cells and blood cells (hemocytes) all embedded in a thick matrix (Nyholm and McFall-Ngai 1998). The host's crypt epithelium undergoes a restructuring whereby the apical surfaces are effaced and blebbed into the crypt spaces in the hours preceding dawn (Wier et al. 2010). There is also an upregulation of greater than 50 cytoskeleton-related genes during this time reflecting dynamic changes to the crypt epithelium. After dawn, the epithelium reestablishes polarity, and there is a concomitant downregulation of the same cytoskeleton-related genes. The symbionts also undergo cyclic transcriptional changes that are notably tied to metabolism. Genes associated with anaerobic respiration of glycerol are upregulated during the day and transition to a metabolism of chitin fermentation in the hours before dawn. Lipid analysis also suggests that host-derived fatty acids are incorporated into the symbionts' membranes as a result of effacement of the crypt epithelium (Wier et al. 2010).

11.4 Steps to Colonization

One of the hallmarks of the squid–vibrio association is the high degree of specificity. *Vibrio fischeri* is the only bacterium that is capable of colonizing the light organ. Work over many years has focused on the intricate communication that occurs between the host and symbiont and the molecular and cellular dialogue that is involved. *V. fischeri* from the environment must colonize the host among a background of a number of nonsymbiotic microorganisms. In areas with large squid populations, *V. fischeri* can be found at concentrations of 100–1500 cells per ml of seawater among a background of a million other nonsymbiotic bacteria (Lee and Ruby 1994). The light organ of the juvenile host is also quite different from the adult and is poised to interact with bacteria from the environment. Notably, the juvenile light organ has elaborate ciliated epithelial fields on its lateroventral surfaces. The fields are comprised of two appendages that form a ring surrounding three pores on either side of the light organ. These cilia begin to beat within seconds after the squid emerge from their egg case and help entrain seawater near the vicinity of the pores. *Vibrio fischeri* must enter these pores and travel down a ciliated duct before entering an antechamber that leads to crypt spaces where colonization occurs (Fig. 11.1). One of the challenges for *V. fischeri* is that the mantle volume of the squid is quite small (1.3 μl) such that only a few *V. fischeri* cells are present in the mantle cavity and any given time. Because the squid is constantly ventilating, *V. fischeri* must find one of the three pores on either side of the light organ before being expelled back into the environment. How does *V. fischeri* overcome this challenge, and what prevents nonsymbiotic bacteria from entering and colonizing the light organ? There are a series of physical, chemical, and cellular barriers that assist the host in selecting the correct symbiont from seawater, and in turn, *V. fischeri* has mechanisms to ensure that it can overcome each of these potential barriers and successfully colonize the light organ.

One way in which the host helps entrain environmental bacteria is the secretion of mucus from the ciliated fields soon after hatching. Gram-negative bacteria from the environment, including *V. fischeri*, are then capable of aggregating in this mucus forming a type of biofilm (Nyholm et al. 2000). In addition to the host mucus, *V. fischeri* is capable of generating a biofilm using an 18-gene *syp* (symbiosis polysaccharide) locus that is important for this purpose (Yip et al. 2005, 2006; Norsworthy and Visick 2013). Association with the biofilm also represents the first site of specificity as *V. fischeri* outcompetes other Gram-negative bacteria in these aggregations (Nyholm and McFall-Ngai 2003). Recent work has shown that only a few cells (three to five) are necessary to aggregate and initiate colonization (Altura et al. 2013). In addition to association with the mucus, these cells also appear to directly bind cilia associated with the surface epithelium before migrating to and entering the light organ pores (Altura et al. 2013). Amazingly, interactions with these few initial cells lead to widespread transcriptional changes in the host that appear to prime the organ for colonization (Kremer et al. 2013). Specifically, the

host upregulates expression of an endochitinase that hydrolyzes polymeric chitin found in the host mucus secretions to chitobiose. Chitobiose, in turn, acts as a chemoattractant for cells of *V. fischeri* that migrate to the pores (Mandel et al. 2012).

The ability to migrate to the pores and away from the initial aggregate is dependent on motility. The mantle cavity of a juvenile squid is a very dynamic microenvironment. The ciliated fields create microcurrents that help to move particles toward the pores, but then outward-beating cilia in the ducts present a challenge for bacteria moving down these ducts to the antechamber within the light organ tissue. *Vibrio fischeri* has a tuft of polar flagella that it uses to move and chemotax toward the ducts (Ruby and Asato 1993). Knocking out various genes involved with motility has demonstrated that both non-motile or hyper-motile mutants are deficient in colonization (Graf et al. 1994; Nyholm et al. 2000; Millikan and Ruby 2002, 2004; Wolfe et al. 2004; Brennan et al. 2013). The symbiont also undergoes morphological changes during its transition from a free-living bacterium to a symbiont. For example, after colonization, *V. fischeri* loses its flagella while it divides and populates the light organ (Ruby and Asato 1993). A proteomics study of the crypt contents showed that a number of symbiont proteins involved with bacterial flagellar production were present during venting, suggesting that *V. fischeri* may be capable of anticipating the transition from the squid host to the environment since the symbiont loses its flagella after colonization (Schleicher and Nyholm 2011). Symbionts that are vented from the light organ each morning are still viable and regain their flagella-based motility.

In addition to motility, chemotaxis is also important in the trek that *V. fischeri* must make to the host. This chemotaxis often relies on two-component regulatory systems consisting of a response regulator and sensor kinase, along with methyl-accepting chemotaxis proteins [MCPs; reviewed in Norsworthy and Visick (2013)]. These MCPs are coupled to a sensor kinase (CheA) and two response regulators (CheY and CheB). Methylation of MCPs allows bacteria to respond rapidly to environmental cues and is regulated by a methyltransferase (CheR) and methylesterase (CheB). *Vibrio fischeri* has a number of putative MCPs (43 predicted from the genome) suggesting that the symbiont can respond to many environmental cues (Brennan et al. 2013). Experimental evidence showed that deletions to either CheY or CheR lead to a deficiency in colonization (Hussa et al. 2007; Deloney-Marino and Visick 2012). *Vibrio fischeri* is also capable of chemotaxing toward *N*-acetylneuraminic acid (NANA) (DeLoney-Marino et al. 2003) and chitin derivatives (Mandel et al. 2012) (GlcNAc and GlcNAc2) which are found in the shed mucus and light organ crypts, respectively (Nyholm et al. 2000; Heath-Heckman and McFall-Ngai 2011). Furthermore, disruption of a chitin gradient prevents *V. fischeri* from entering the light organ pores (Mandel et al. 2012).

11.5 Molecular Dialogue Between the Partners

Much of the molecular "conversation" between the partners is mediated by what are known as microbe-associated molecular patterns (MAMPs) (Koropatnick et al. 2004) (Table 11.1). These are molecules unique to microbes and include, for example, the cell wall component peptidoglycan, outer-membrane proteins, or other compounds like lipopolysaccharide (LPS). In turn, these MAMPs are often detected by host pattern recognition receptors (PRRs), the binding of which results in downstream signaling cascades that influence host transcription, often related to the immune system (Nyholm and Graf 2012). One of the first MAMPs that *E. scolopes* encounters is bacterial peptidoglycan (PGN). The host hatches from its egg case into seawater containing numerous environmental bacteria (up to 10^6 cells/ml). Since both Gram-positive and Gram-negative bacteria have PGN, it is quite abundant in seawater. Exposing hatchling squid to bacteria or exogenous PGN leads to mucus secretion from the ciliated epithelium of the nascent light organ (Nyholm et al. 2002). One area of intensive research in the squid–vibrio association is the influence of the symbiont on the development of the light organ. Early observations showed that colonization by *V. fischeri* leads to morphogenesis of the light organ whereby ciliated epithelial tissues that assist in colonization undergo apoptosis and regress over the first days of the host-symbiont association (McFall-Ngai and Ruby 1991; Montgomery and McFall-Ngai 1994; Doino and McFall-Ngai 1995; Foster and McFall-Ngai 1998). Two MAMPs produced by *V. fischeri*, LPS and a PGN derivative called tracheal cytotoxin (TCT), work synergistically to induce this morphogenesis (Foster and McFall-Ngai 1998; Koropatnick et al. 2004). These findings are significant as these MAMPs are often associated with inducing virulence in pathogenic associations. For example, TCT had been only known in the human pathogens *Neisseria gonorrhoeae* and *Bordetella*

Table 11.1 Host and symbiont mediators of specificity and persistence in the squid–vibrio association

Host mediators of specificity and persistence	Symbiont mediators of specificity and persistence
Innate immune system – Hemocytes (trafficking to light organ, tolerance to *V. fischeri*, delivery of chitin to symbionts) – Pattern recognition receptors (Toll-like receptors, TLRs; peptidoglycan recognition proteins; PGRPs, galectins, lipid-binding proteins, LBPs – Reactive oxygen and nitrogen species – Galaxins – Cryptochromes – Hemocyanin Alkaline phosphatases Specialized ciliated epithelium Host mucus secretions Endochitinase	Microbe-associated molecular patterns (MAMPs) – Lipopolysaccharide (LPS) – Peptidoglycan (PGN) – Tracheal cytotoxin (TCT) Light production (dark mutants do not persist in light organ) Motility Biofilm formation (Syp locus) Chemotaxis (CheR)

pertussis where it promotes virulence by damaging epithelial cells. The structure of TCT in *V. fischeri* is identical yet it does not cause virulence in the squid. These data suggest that what would normally be classified as a toxin may also serve as a signaling molecule during normal development.

Euprymna scolopes has a number of pattern recognition receptors (PRRs) that have the potential to respond to MAMPs. These include five peptidoglycan recognition proteins (PGRPs), a Toll-like receptor (TLR), and LPS-binding proteins (LBPs) (Goodson et al. 2005; Troll et al. 2009, 2010; Krasity et al. 2011; Collins et al. 2012b). Host PGRPs are expressed in specific tissues and cell types, and some appear to change upon colonization. For example, EsPGRP1 is present in the nuclei of host epithelial cells but is later absent in cells that undergo apoptosis during light organ morphogenesis (Troll et al. 2009). Mutants of *V. fischeri* that are defective in TCT release do not induce loss of EsPGRP1. The EsPGRP2 protein has a secreted form that has been found in the light organ crypt spaces as well as in mucus produced during the onset of colonization (Troll et al. 2010). This same protein, EsPGRP2, also has an amidase activity enabling it to degrade TCT. In addition, the host has two alkaline phosphatases (APs) that are present in the light organ and have the ability to dephosphorylate and inactivate the lipid A portion of *V. fischeri* LPS (Rader et al. 2012). Twelve hours postcolonization, a time point when light organ morphogenesis is irreversible (Doino and McFall-Ngai 1995), there is an increase of AP expression in the light organ that mirrors the daily rhythm of the symbiosis, higher in the evening and lower during the day. Taken together these data suggest that the host has mechanisms to respond to *V. fischeri* MAMPs in order to promote colonization and then to maintain the association.

The production of light by *V. fischeri* serves as the basis of the squid–vibrio association. The effectiveness of counterillumination is difficult to demonstrate outside a controlled experiment in the presence of a predator, but the importance of light production by the symbiont and the ability of the host to detect that light are strongly supported. One question that is often posed is how the host prevents a "cheater," signifying in this case bacteria that consume resources but do not provide illumination, from colonizing the light organ. Is it possible for a "dark" strain of *V. fischeri* to colonize the light organ, effectively parasitizing the host without having to produce light? An experimental study showed that a strain of *V. fischeri* that is deficient in producing light was unable to persist in the light organ (Visick et al. 2000). Furthermore, mutants defective for light production are impaired in terms of inducing developmental phenotypes normally associated with colonization (Chun et al. 2008; McFall-Ngai et al. 2012). Light produced by the symbiont also appears to lead to an increase in expression of PRRs and innate immunity factors like EsPGRP1, LPS-binding protein, and galaxins (Chun et al. 2008). These data suggest that the host has the ability to detect and respond to light produced by the symbiont. How is this achieved? In some ways the light organ very much resembles an eye. There are a lens and reflective tissue that help to transmit the light produced by the symbionts. In addition, the light organ tissues of the host express the genes necessary for phototransduction including opsin, rhodopsin kinase, and arrestin (Tong et al. 2009), and colonization by *V. fischeri* subsequently influences the

expression of host genes associated with eye specification and development (Peyer et al. 2014). The host also has two cryptochromes, proteins that in other systems help regulate circadian rhythms (Heath-Heckman et al. 2013). Expression of one of these, *escry1*, had an expression pattern that mirrored the daily rhythm of bacterial bioluminescence, being highest when luminescence was also at its peak. Colonization by a bacterial mutant defective in light production was noted to disrupt the rhythm of *escry1* expression, and *escry1* expression subsequently was capable of being rescued by exposure of the squid to blue light. Interestingly, exposure to just blue light and the MAMP TCT and lipid A also led to rhythmic *escry1* expression. Therefore in the squid–vibrio association, light acts as a signal and morphogen similar to the MAMPs described above. How does the host sanction "dark" cheaters? This question remains to be answered but given the importance of light production to the host's survival, having the ability to regulate and respond to the symbiont's light is paramount.

11.6 Interactions with the Innate Immune System

All organisms have mechanisms to defend themselves against pathogenic microorganisms (usually bacteria, viruses, or eukaryotic parasites). The immune system often mediates these interactions and much research has been focused on understanding how hosts overcome these challenges. However, a more recent view of the immune system suggests that it also has a significant role in mediating the establishment and maintenance of beneficial associations. Having a need to interact and "communicate" with environmental microorganisms was likely a major driving force during the evolution of immune systems, and studies have shown that the immune system plays a critical role in mediating symbioses with microbes (Nyholm and Graf 2012).

Invertebrates lack canonical adaptive immunity meaning they don't have the capability to produce antibodies to specific antigens (this trait appeared with the jawed vertebrates). Alternate mechanisms that generate highly variable immune proteins have been described for some specific organisms [e.g., fibrinogen-related proteins in the snail *Biomphalaria glabrata* (Zhang et al. 2004) or Down's syndrome cell adhesion molecules in *Drosophila melanogaster* (Watson et al. 2005)]. However, most invertebrates are thought to rely on the innate immune system, primarily comprised of phagocytes and the production of antimicrobial compounds or reactive oxygen and nitrogen species. How is it that invertebrates can form highly specific associations with microorganisms in the absence of antibody-based immunological memory? Many studies have shown that the innate immune system of animals can interact specifically with microorganisms to form these associations [reviewed in Nyholm and Graf (2012)].

The cellular-based immune system of cephalopods (squid, octopuses, and cuttlefish) largely consists of macrophage-like hemocytes (Fig. 11.3). Hematopoiesis of these cells occurs in a specialized organ called the white body from which mature

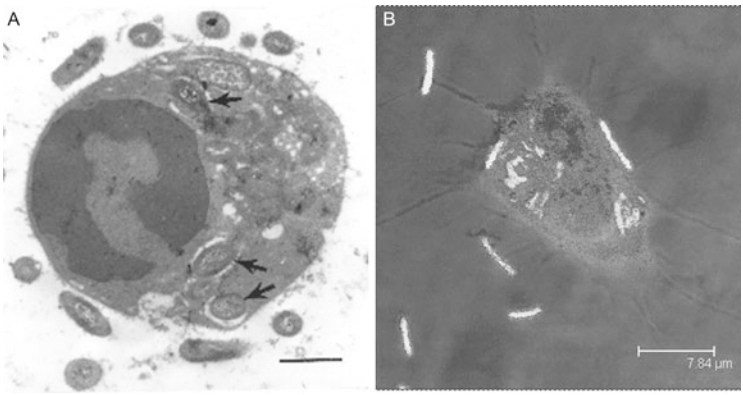

Fig. 11.3 Interactions with host hemocytes. Specificity is partially mediated by the main innate immune cell type of the host, the macrophage-like hemocyte. (**a**) Hemocytes are sometimes observed with phagocytosed bacteria (*arrows*) within the light organ crypt spaces. (**b**) The ability for hemocytes to differentially bind symbiotic and nonsymbiotic bacteria is influenced by colonization of the light organ. Removing the symbiont by curing the host with antibiotics leads to increase binding of *V. fischeri* (Nyholm et al 2009) [(**a**) Adapted from Nyholm and McFall-Ngai (1998)]

hemocytes enter the circulatory system. Unlike other molluscs, cephalopods have a very complex and enclosed circulatory system with a systemic and two branchial hearts that distribute hemolymph containing the main oxygen-binding protein hemocyanin, other extracellular proteins, and hemocytes to all of the tissues and organs. The light organ is also highly vascularized, and hemocytes are capable of migrating to and entering the crypt spaces that house *V. fischeri*, and thus the hemocytes are expelled with the daily venting of the symbionts each morning (Nyholm and McFall-Ngai 1998). Why do hemocytes migrate into the light organ? Are they involved with regulating the symbiosis, perhaps preventing overgrowth of the symbiont or sanctioning strains of *V. fischeri* that are inefficient or underperforming in light production? Because the crypt spaces are directly linked to the environment via the pores and ciliated ducts, the potential for other nonsymbiotic or pathogenic microorganisms to enter the crypts is always present. Observations of symbiotic juveniles have shown that hemocytes in the crypt spaces occasionally have engulfed or phagocytosed bacteria (Nyholm and McFall-Ngai 1998). This observation begs the question: are these phagocytosed bacteria *V. fischeri* or nonsymbiotic bacteria that have made their way into the light organ and are in the process of being removed?

To understand the dynamics of this process, in vitro binding assays showed that host hemocytes that were isolated from colonized adult animals recognized and bound the cells of four marine bacterial species to different degrees (Nyholm et al. 2009). Specifically, hemocytes bound significantly more cells of *Vibrio harveyi* and *Photobacterium leiognathi* than those of *V. fischeri* and *Vibrio parahaemolyticus*. To examine whether hemocyte binding behavior was influenced by colonization, the symbiont population was eliminated from a subset of animals

by antibiotic treatment prior to the collection of the hemocytes. A cohort of animals was maintained in the cured state for an additional 4 days, during which time bacteria-binding efficiency was determined for hemocytes isolated either from cured (naïve) or symbiotic animals. No change was detected in the ability of the hemocytes from symbiotic hosts to bind any of the three bacterial strains over the 5-day experiment; however, by day 4, the ability of the naïve hemocytes to bind *V. fischeri* cells had become significantly greater, increasing to fivefold by 5 days. This increased binding activity was specific toward *V. fischeri* as binding to *V. parahaemolyticus* or *V. harveyi* was unchanged.

These results show that the ability to avoid adherence to hemocytes of *E. scolopes* varied among related bacterial species, and the hemocytes' response was specifically altered by the colonization state of the light organ. These data suggest that colonization can lead to a type of host immune tolerance to *V. fischeri*. The mechanisms by which *V. fischeri* avoids adherence and induces this tolerance remain to be characterized, but experimental data show that a *V. fischeri* outer-membrane protein (OmpU) may be involved as deletion of OmpU leads to a significant increase in binding to hemocytes. Ultimately we'd like to understand the molecular mechanisms by which the hemocytes can distinguish between symbiotic and nonsymbiotic bacteria. Research in the Nyholm lab is currently focused on understanding changes in hemocyte gene expression and protein abundance in response to colonization. A number of genes and proteins from hemocytes have been identified that are predicted to be involved with immune response, including the detection of MAMPs and downstream signaling. Furthermore, colonization state has been found to influence hemocyte gene expression of the PRR EsPGRP5, nitric oxide synthase (NOS), and a squid orthologue of the complement component C3 (Collins et al. 2012b) as well as protein abundance of a number of factors involved with the cell skeleton, lysosomal function, and other components of innate immunity (Schleicher et al. 2014).

In addition to their traditional role in immunity, hemocytes appear to play other functions in the light organ. As mentioned earlier, there is a pronounced diel rhythm whereby 95 % of the symbionts are expelled each morning at dawn. Hemocytes and shed epithelial cells are also expelled with this exudate. An analysis of both host and symbiont transcription over the diel cycle suggests that *V. fischeri* switches between oxidative phosphorylation during the day to chitin fermentation at night (Wier et al. 2010). Where does this chitin come from? A survey of light organ tissues revealed that only hemocytes contain chitin and it is often in cytoplasmic granules (Heath-Heckman and McFall-Ngai 2011). Therefore hemocyte migration into the crypt spaces may also serve the purpose of providing a source of chitin to *V. fischeri*. Hemocyte trafficking is also influenced by early exposure to the symbiont and may be involved with early morphogenesis. After hatchling squid are exposed to *V. fischeri* for 2 h; hemocytes traffic into the ciliated epithelial fields that will then undergo apoptosis and regression 4 days after colonization (Koropatnick et al. 2007). One area of future research is to understand the extent to which hemocytes traffic into the light organ and whether they then migrate out into the circulatory system. Could *V. fischeri* or bacterial MAMPs have a more

systemic effect on the host (e.g., by influencing other organs as has been shown for gut bacteria in mammals), and do hemocytes act as a potential mediator for signaling to other tissues? These questions remain to be answered.

In addition to cellular-based immunity, the host also has a number of other classical components of the innate immune system including reactive oxygen and nitrogen species (Tomarev et al. 1993; Weis et al. 1996; Small and McFall-Ngai 1999; Schleicher and Nyholm 2011). A squid halide peroxidase (sHPO) is present in the ducts and crypt spaces of the light organ (Weis et al. 1996). This enzyme converts hydrogen peroxide (H_2O_2) into hypohalous acid (HOCl) against which bacteria are not known to have an effective defense. To overcome this challenge, *V. fischeri* has a periplasmic catalase that effectively converts hydrogen peroxide to water and oxygen thus preventing production of hypohalous acid (Visick and Ruby 1998). A mutation in the gene that encodes this catalase (katA) was associated with both sensitivity to H_2O_2 and defective colonization when competed against wild-type *V. fischeri* (Visick and Ruby 1998). A proteomics study of the light organ exudate also detected the antioxidant enzymes alkyl hydroperoxide reductase (AhpC) and thioredoxin-dependent thiol peroxidase (Bcp) in addition to KatA (Schleicher and Nyholm 2011). So far, it is unclear whether these additional antioxidant enzymes play a role in the symbiosis.

In addition to ROS, the host also has a nitric oxide synthase (NOS) that is capable of producing nitric oxide (NO). NO along with other antimicrobial factors like PGRP2, chitinases, lysozyme, and proteases are secreted in the mucus of the host (Kremer et al. 2014; Troll et al. 2010). Colonization induces an attenuation of NOS and NO, and the *V. fischeri* MAMPs LPS and TCT can together induce this effect (Davidson et al. 2004; Altura et al. 2011). *Vibrio fischeri* has a homologue to a heme NO/oxygen-binding (H-NOX) protein that may help mediate responding to NO but is also linked to iron uptake for the symbiont (Wang et al. 2010; Wang and Ruby 2011). Other compounds including a flavohemoglobin, an alternative oxidase (AOX), and NO-responsive regulatory protein (NsrR) also likely play a role in protecting *V. fischeri* from the effects of NO (Wang et al. 2010; Dunn et al. 2010).

The host harbors a number of other potentially antimicrobial compounds. For example, the oxygen-binding protein hemocyanin is found in the crypt spaces of the light organ. In addition to likely serving as a means to deliver oxygen to the symbionts for bioluminescence, hemocyanin has antimicrobial activity and is present in the mucus secreted by ciliated fields during colonization and may play a role in recruitment of *V. fischeri* (Kremer et al. 2014). Analyses of the transcriptome and proteome of light organs and hemocytes also revealed a number of putative members of the complement cascade often associated with the innate immune system of other animals (Castillo et al. 2009; Collins et al. 2012b). An orthologue of the complement component C3 was localized to the light organ crypt epithelium and in hemocytes, and that orthologue of C3 was differentially expressed in blood cells from both symbiotic and cured (antibiotic-treated) hosts. The hemocyte proteome also revealed a number of thioester-containing proteins (TEPs) that may be involved in a complement-like response.

Recently, the role of a protein known as galaxin was characterized in the squid–vibrio association (Heath-Heckman et al. 2014). Galaxins are a group of proteins that have been described in other hosts (corals and hydrothermal vent tubeworms), yet their role in symbiosis is poorly understood. A gene expression study showed that in *E. scolopes*, galaxin is upregulated after colonization of the light organ (Chun et al. 2008) and follows the daily rhythm in adult light organs (Wier et al. 2010). The protein EsGal1 is the dominant galaxin in the light organ and expression of this protein can be partially induced by the MAMPs TCT and Lipid A (Heath-Heckman et al. 2014). The EsGal1 protein is also found on the surface of the light organ epithelium and in mucus secreted during initial aggregation. In vitro assays have shown that EsGal1 inhibited the growth of Gram-positive bacteria and therefore may be involved with the initial selection of the symbiont since Gram-positive bacteria are excluded from aggregations that form outside the light organ during colonization (Heath-Heckman et al. 2014; Nyholm et al. 2000; Nyholm and McFall-Ngai 2004).

11.7 Developing *E. scolopes* as a Model for Both Binary and Consortial Symbioses (Accessory Nidamental Gland Association)

A common yet poorly understood animal–bacterial association occurs between members of squid and cuttlefish species and bacterial consortia that reside within a reproductive gland of female hosts called the accessory nidamental gland (ANG) (Bloodgood 1977). This highly pigmented organ of the reproductive tract of sexually mature females harbors a dense consortium of bacteria (Fig. 11.4). Research in several squid species shows that these bacteria are housed in epithelium-lined tubules that are attached to the nidamental gland, the organ that secretes the jelly coat surrounding fertilized eggs, and these bacteria are deposited into the egg cases (Barbieri et al. 2001; Collins et al. 2012a). Many squid lay their eggs in clutches or masses on the seafloor bottom where they must develop over a period of approximately 1 month. During this time, the developing embryos are exposed and unprotected. Although the role of these bacteria is unclear, they may help prevent both unwanted fouling from other microorganisms and predation. Culture-dependent and independent methods have identified the dominant members of bacteria that are housed in cephalopod ANGs. In *E. scolopes*, as with other cephalopod species, the ANG is dominated by *Alphaproteobacteria*, usually members of the *Roseobacter* clade within the *Rhodobacterales*, commonly found in marine environments (Collins et al. 2012a). Other contributing taxa include members of the *Bacteroidetes*, *Gammaproteobacteria,* and *Verrucomicrobia* (Fig. 11.4). Efforts are underway to understand the role that this consortium plays in the reproduction of *E. scolopes* but there are some common themes that have also been observed in the light organ association, such as housing a dense bacterial

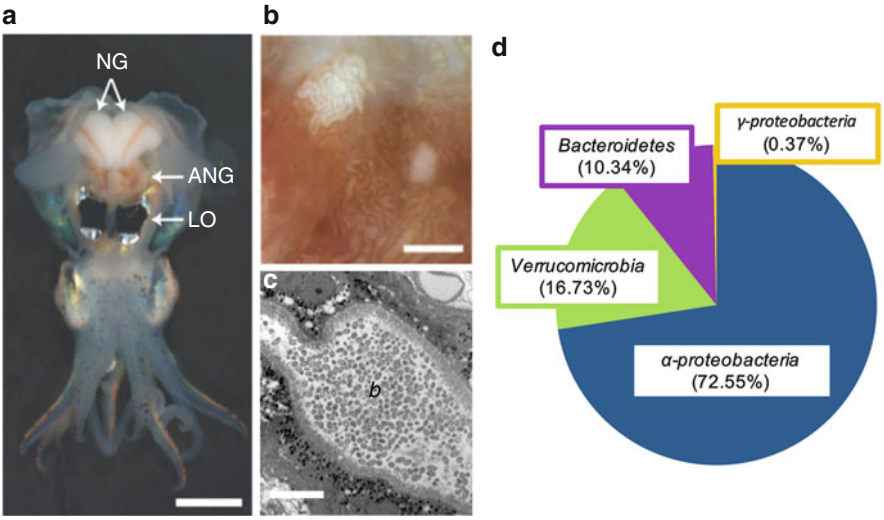

Fig. 11.4 The accessory nidamental gland symbiosis of *E. scolopes*. (**a**) Ventral dissection showing the position of the nidamental gland (NG) and accessory nidamental gland (ANG) in relation to the light organ (LO). Scale, 5 mm. (**b**) Close-up of ANG showing pigmented tubules containing bacteria. Scale, 0.5 mm. (**c**) Electron micrograph showing bacteria (b) within host tubules. Scale, 5 μm. (**d**) Results of sequencing of the 16S ribosomal gene from the ANG of *E. scolopes*. The communities had a number of members of the *Rhodobacterales*, similar to findings for other squid species. Members of the *Verrucomicrobia* and the *Bacteroidetes* phyla were also prominent members. The ANG and light organ association in *E. scolopes* will allow researchers to explore both a binary and consortial symbiosis in the same host [Adapted from Collins et al. (2012a); Copyright © American Society for Microbiology]

population in epithelium-lined crypt spaces and the trafficking of hemocytes into the crypts (Collins et al. 2012a). The same sHPO that was found in the light organ is also present in the ANG (Small and McFall-Ngai 1999), and a second galaxin (EsGal2) has higher expression in the ANG when compared to the light organ (Heath-Heckman et al. 2014).

11.8 Conclusions and Future Directions

The squid–vibrio association has provided a wealth of information on how beneficial bacteria interact with animal hosts. Because of the binary nature of the association and the fact that each partner can be maintained independently, a broad number of experimental approaches have been applied successfully to study the squid host and its symbionts. However, no model system is ideal and each faces its own set of challenges. One thing that may further contribute to the use of the squid–vibrio association among researchers will be the development of more tools on the host side. For example, unlike other model hosts such as *Drosophila*

melanogaster, zebrafish, and mice, genetic knockout and knockdown techniques have not yet been developed in the squid. Also, while there is a growing body of transcriptome information available for *E. scolopes*, the genome itself has not been sequenced although efforts are underway. The present lack of a full genomic sequence for this squid species has made identifying its genes and proteins from proteomic studies to be an unnecessarily difficult task. Cephalopod genomes have posed significant challenges for sequencing as they tend to be quite large (*E. scolopes* is 3.7 Gb) and have abundant repetitive sequences (Albertin et al. 2012). Most of the colonization experiments in the squid–vibrio association are carried out over the first 5 days after the squid hatch. Although methods for raising the squid to maturity have been described, it is often a labor intensive and time-consuming process (Hanlon et al. 1997; Lee et al. 2009). Recent efforts with aquaculture of the host, however, are opening up new and exciting avenues of research (Koch et al. 2014). By reliably raising the squid, the research community can analyze the long-term developmental effects of colonization. On the symbiont side, the ability to mutagenize *V. fischeri* has always been a powerful tool for understanding the effects of specific genes on the symbiosis (Ruby 2008). Comparative genomics between native and nonnative *V. fischeri* strains has also proven valuable for understanding the genes as well as the metabolic and physiologic processes that are important for host-symbiont specificity (Mandel et al. 2009). The diel rhythm of the squid–vibrio association and the ability to passage bacteria from one host to another are also proving to be an important asset that has allowed researchers to apply experimental evolution techniques to understand traits that are important for colonization (Schuster et al. 2010; Soto et al. 2012).

Acknowledgments I would like to thank the numerous collaborators who have contributed to this research, especially the McFall-Ngai and Ruby laboratories at the University of Hawaii. Also, research in the squid–vibrio association would not be possible without the generous support of the Kewalo Marine Laboratory and Hawaii Institute of Marine Biology at the University of Hawaii. I'd like to thank A. Suria for the contribution of drawings of the host and symbiont. This work was funded by NSF IOS 0958006 and the University of Connecticut Research Foundation to SVN.

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