The Importance of Microbes in Animal Development: Lessons from the Squid-Vibrio Symbiosis

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Keywords

Abstract

Developmental biology is among the many subdisciplines of the life sciences
being transformed by our increasing awareness of the role of coevolved mi-
crobial symbionts in health and disease. Most symbioses are horizontally
acquired, i.e., they begin anew each generation. In such associations, the em-
bryonic period prepares the animal to engage with the coevolved partner(s)
with fidelity following birth or hatching. Once interactions are underway,
the microbial partners drive maturation of tissues that are either directly
associated with or distant from the symbiont populations. Animal alliances
often involve complex microbial communities, such as those in the vertebrate
gastrointestinal tract. A series of simpler-model systems is providing insight
into the basic rules and principles that govern the establishment and main-
tenance of stable animal-microbe partnerships. This review focuses on what
biologists have learned about the developmental trajectory of horizontally
acquired symbioses through the study of the binary squid-vibrio model.

Contents

INTRODUCTION—IT'S A NEW DAY FOR DEVELOPMENTAL BIOLOGY

Through recent technological advances, biologists have come to recognize the extent to which animals and plants rely on microbes for the success of their developmental programs (25, 26, 52). For example, fascinating new molecular data demonstrate that many, perhaps most, marine invertebrates require transient, microbe-derived chemical cues, often associated with biofilms, to progress from one developmental stage to the next (30, 82). In such cases, it is likely that, whereas the animal experiences selection pressure to recognize a particular microbial cue, the fitness of the microbe may not be specifically affected. In contrast, selection pressure on the specificity of both partners is a characteristic of mutualistic symbioses (see sidebar, The Power of Words). However, unlike the monospecific legume-rhizobia associations (for reviews see 36, 68), the molecular details of such recognition are generally not well understood in animals, where microbes occur primarily as complex consortia. Only with advances in nucleic acid technology over the last decade could biologists determine the microbial composition of such communities and study the development-inducing activities of its members (6, 26). Relevant here is the recognition that often the organism undergoing development is not only the host but rather a set of interdependent organisms, in persistent symbiosis, whose developmental programs have coevolved. These new findings add exciting dimensions to the field of animal developmental biology and promise to bring an unprecedented integration of microbiology into this discipline.

THE POWER OF WORDS

As the field of host-microbe interactions grows at a fierce rate, considerable controversy about, variation in, and misuse of, terminology has understandably crept into the field of symbiosis. For clarity, I define here how I apply the key terms. I use the simple, classical definition of symbiosis, established by de Bary in 1879 (14), i.e., organisms living together. The primary types of symbioses, i.e., mutualism, commensalism, and parasitism (or pathogenesis), refer to a state or outcome. In mutualistic symbioses, the fitness of both partners, i.e., their ability to contribute to the next generation, is enhanced; in commensal relationships, one partner benefits while the other is unaffected; and in parasitic associations, one partner's fitness is positively affected while the other's is harmed. Because these states are context specific, the terms do not refer to an organism itself but rather the state of the interaction; whether the symbiosis is one type or another depends on outcome (72). When the effects of fitness on the partners is difficult to define, as is often true in complex systems, the most accurate word to describe the association is symbiosis, a higher-level term with no designation of fitness.

Developmental biologists studying symbiosis seek to define how the holobiont, i.e., the co-evolved partners in a symbiosis (99), persists through generations as a unit of selection. Microbial symbionts are either vertically transmitted, i.e., passed maternally in or on the egg (e.g., insect-bacteriocyte symbionts) or horizontally transmitted, i.e., acquired anew from the environment (e.g., gut consortia) (9). Terrestrial animals, such as social insects (22) and mammals (34), often have a facilitated horizontal transmission, in which symbionts are acquired through interactions with more mature conspecifics (19). No matter the mode of transmission, all symbiotic associations must alter developmental programs to accommodate the partnership, although several salient differences between vertical and horizontal transmission are noteworthy. For example, vertical transmission, by definition, ensures maintenance of the coevolved symbiont(s) with fidelity. Thus, no need exists for mechanisms that promote specificity during the initiation of symbiosis in each successive generation. In contrast, during horizontal transmission, the naive juvenile host is exposed not just to the appropriate symbiont(s) but also to an array of other environmental microbes. Thus, mechanisms must be present in the host, the symbionts, or both to facilitate the establishment of the right partnerships to the exclusion of interlopers, and such mechanisms must be integrated into the developmental programs of all members.

In addition to mode of transmission, the patterns of development are also influenced, for one or more partners, by where along the spectrum of obligate to facultative a particular symbiosis lies. At one end of the spectrum, strong selection over evolutionary time has resulted in unequivocally obligate mutualisms. For example, in the insect-bacteriocyte associations, both partners have a strict requirement for the relationship and rarely occur independent of each other, even under laboratory conditions (18). Conversely, in obligate parasitic symbioses, such as between many insects and *Wolbachia*-type organisms, it is only the microbial partner that cannot survive without the host. Some situations, however, are more context dependent. For example, only under conditions of low nitrogen in the soil do leguminous plants entertain the development of a symbiosis with nitrogen-fixing rhizobia, a process that is tightly controlled through gene regulation in the plant and bacterium (73). In all cases examined, diverse strategies have evolved that incorporate into the host's developmental program an accommodation to the form and function of the specific symbiosis.

In this review, through the lens of the squid-vibrio light-organ association, I discuss the developmental trajectory of a horizontally acquired symbiosis. Recent reviews (63, 83) on the biology of the symbiont *Vibrio fischeri* provide a detailed, up-to-date summary of what is known about *V. fischeri* as a species, both in symbiosis as well as in other ecological niches. Here, I focus my efforts on a review of what is known about the development of the symbiosis, the interplay between partners that mediates this developmental program, and the near and far horizons of the system.

THE BASIC NATURE OF THE SQUID-VIBRIO SYMBIOSIS AND THE ENDPOINT OF DEVELOPMENT, THE MATURE ASSOCIATION

The symbiosis between the squid *Euprymna scolopes* and the luminous bacterium *V. fischeri* appears to be obligate for the host in nature but not for the microbial partner. (See **Supplemental Figure 1**: Follow the **Supplemental Material link** in the online version of this article or at **http://www.annualreviews.org** for a discussion of the phylogenetic position of the partners and the worldwide patterns of squid-vibrio symbioses.) The juvenile animals are colonized shortly after hatching, and all examined animals from wild populations harbor the symbiont in their light organ. *V. fischeri*, however, has a distinct plankton phase independent of the animal. The light produced by the symbiont population is thought to be used by the host animal as an antipredatory camouflage (35, 54), called counterillumination. In this behavioral strategy (98), the animal emits ventral

▶ Supplemental Material

luminescence to mimic down-welling moonlight and starlight. The importance of biolumines-
cence to the host's biology is reflected in the developmental program, which has a built-in
requirement for symbiont luminescence. This incorporation of symbiont light production into de-
velopment underscores the host's imperative to obtain and maintain a luminous microbial partner.

By beginning our discussion with the mature symbiosis, we can best understand the goal
of the association's program of development. Horizontally acquired symbioses typically undergo
postembryonic differentiation in which the host tissues mature and the bacterial symbionts become
stable partners (56). For example, the immune system and microbiota of humans undergo changes
over the host's first few years of life that lead to a mature immune system and stable, resilient set
of symbiont communities in various body sites (33, 50, 70). Studies of mouse development have
demonstrated that the adult state results from a complex reciprocal interaction among the partners
(74, 78, 88). The maturation of such symbioses can result in either morphologically elaborate
regions of host-microbe interaction, such as the rumen, or more subtle yet critical relationships,
such as in the human integument (29).

The developmental program of the squid light organ produces a dedicated, complex bilobed
structure in the center of the mantle cavity (**Figure 1a**). These organs show strong convergence
in form and function with the eye (**Figure 1b**; 54, 86). Instead of the photoreceptive tissue of the
retina, the interior epithelial layers of the light organ are rendered photogenic by an extracellular
symbiont population that resides in crypts lined by complex microvilli (**Figure 1c**). To modify
the incident light, each lobe of the organ has (*a*) a reflective layer (or tapetum), with imbricated
layers of the protein reflectin (12), which serve to direct light; (*b*) a choroid/iris-like pigmented
layer that controls the direction and amount of light emitted (54); and (*c*) a muscle-derived lens
that achieves transparency by high concentrations of the enzyme aldehyde dehydrogenase, one of
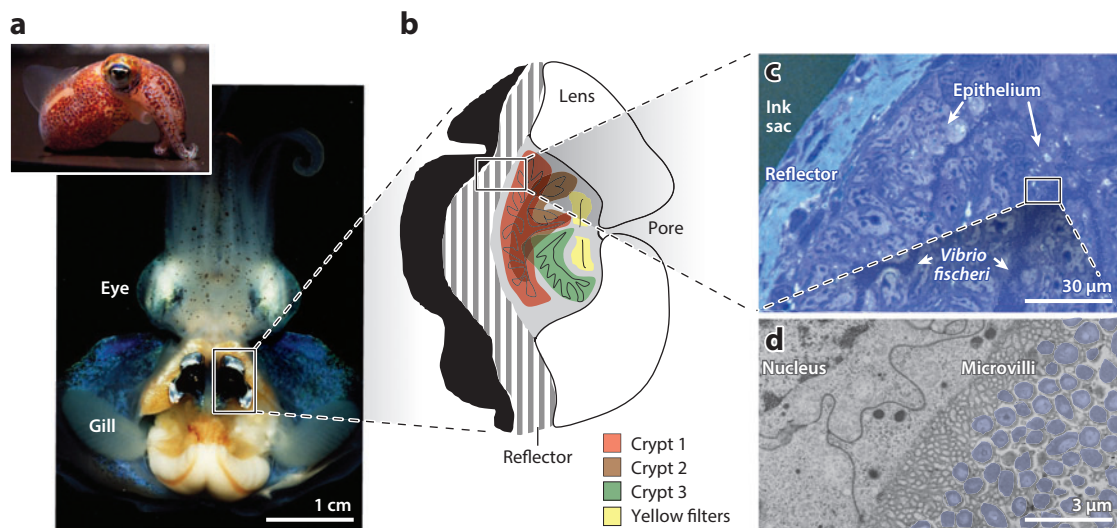

Figure 1

The mature symbiosis. (*a*) A ventral dissection of the adult animal reveals the bilobed light organ in the center of the mantle (body)
cavity. (*b*) A frontal section through one lobe of the organ illustrates the relationship of the three crypts, which house the symbionts, to
the surrounding light-modulating tissues. The black box is the region of tissue in the histological section (*c, upper right*). (*c, upper*) A
low-magnification view of the tissues shows each *Vibrio fischeri* cell of the symbiont population either in direct contact with host
epithelial cells or only a few cell-lengths away. The white box is the region of the transmission electron micrograph (*lower right*).
(*d, lower*) A high-magnification view shows *V. fischeri* cells (false color, *purple*) in intimate association with complex, lobate microvilli of
the host cell surface.

two enzyme-crystallins of the squid eye (59). (For further discussion of convergence between the light organ and eye, see **Supplemental Material**.) Between the lens and the photogenic tissues, just inside each lobe's single pore, are two yellow filters. Although their function has not yet been determined in the squid light organ, in other bioluminescent animals such yellow filters will red-shift the luminescence, presumably to match the color of down-welling light more effectively (16). Although features of the crypt epithelium itself are influenced by *V. fischeri*, studies of late postembryonic development have shown much of the maturation of the light organ is hardwired; i.e., both the organ's adult shape and the elaboration of its light-controlling tissues are achieved, either in the presence or absence of the symbiont.

In addition to colonizing the surface of the crypt epithelium, *V. fischeri* cells also interact with a small number of host blood cells found within the crypt spaces. These hemocytes are macrophage-like cells and are the only type of blood cells in squids. How the hemocytes enter the crypts is not understood, nor is their function(s) at this site. Nevertheless, histological analysis suggests that the hemocytes migrate between the cells of the epithelium (personal observation), much like the diapedesis of vertebrate blood cells through capillary walls (77). Although it is unclear how long these migrating hemocytes remain in the crypt spaces, at most their residence time is limited by the expulsion every morning that discharges them along with most of the symbionts (28, 65) in a protein-rich matrix (79).

The vast majority of the hemocytes are found circulating in the bloodstream (i.e., hemolymph), and analysis of this population has revealed significant behavioral and molecular differences depending on whether they are obtained from symbiotic or nonsymbiotic adult animals (67). These differences suggest the presence of a symbiont-driven maturation process in the cellular component of the host immune system. For instance, hemocytes extracted from the hemolymph of symbiotic animals bind *V. fischeri* cells less well than hemocytes from noncolonized animals, whereas these two hemocyte populations bind other bacterial species equivalently. Interestingly, this gain of resistance is not seen in a *V. fischeri* mutant defective in OmpU (67), the major outer membrane protein that also mediates pathogenesis in other *Vibrio* spp. (20, 51). Hemocytes from symbiotic and nonsymbiotic animals also show differential gene expression, notably of genes encoding portions of the innate immune system (79). Although the basis of this apparent education of the hemocytes by its specific symbionts is unknown, determining where and how it occurs may provide clues. Because of their large numbers, as well as the daily expulsion process, it seems unlikely that each circulating hemocyte must be educated by passing through the crypt spaces and directly interacting with the symbionts. Although some crypt hemocytes might reenter the bloodstream, carrying information to the circulating population, a more likely mode of education would result from the uptake and transport of bacterial products into the hemolymph. Such metabolomic cues could act at a distance on the circulating hemocytes in a manner analogous to the education of T cells in the thymus by the colonic microbiota of mammals (45). Clearly, the host hemocytes, as well as other aspects of the relationship between the innate immune system and the symbiont population, offer exciting subjects for future exploration.

DAILY OSCILLATIONS IN THE ASSOCIATION

Everything in the universe has a rhythm. Everything dances.
—*Maya Angelou*

This quotation touches on a feature well documented in the biological world but poorly understood as a force governing bacterial symbioses. The members of all phylogenetic domains of the biosphere have rhythms that are entrained by natural cycles, most often those that are circadian.

Circadian rhythms are synchronized in two major ways: through transcriptional and metabolic activities (4). In the former, the clock genes and their relatives regulate transcription associated with different day-to-night activities; in the latter, a metabolic oscillator linked to an organism's rhythm of nutrient utilization is driven by the cyclic oxidation and reduction of peroxiredoxin enzymes. Whereas genes controlling transcriptional oscillation are not well conserved (69), the peroxiredoxins are homologous and play a role in the metabolic rhythms of all organisms (21, 84). As biologists gain evidence of the profound influences of the microbiota on mammalian metabolism and integrate that information with well-documented daily metabolic rhythms of the host, it would not be surprising to find that host-symbiont interactions are under circadian control. Clock-gene oscillations have been documented in both the mucosal immune system and the epithelium of the gut (10). Whether transcriptional activities of the associated microbiota are affected by these rhythms remains to be determined, but recent data have demonstrated that microbe-associated molecular pattern (MAMP)/Toll-like receptor interactions that maintain homeostasis of the gut epithelium require normal circadian oscillations (62).

The squid-vibrio system is the only animal-bacterial symbiosis whose daily rhythms have been studied extensively. Early on, it was discovered that the per-cell luminescence of symbiotic *V. fischeri* colonizing juveniles varies over the day-night cycle (5). The highest luminescence levels occur in the early evening as the animal emerges to forage in the water column, whereas the lowest levels are in the hours before dawn. Subsequently, the daily expulsion of most of the crypts' contents into the surrounding seawater was observed (28).

In response to these findings, a study of the transcriptome of the adult crypt epithelium and its associated symbiont population was conducted at 6-h intervals over the 24-h day-night cycle. The data revealed a complex, daily dialogue between the partners (94). In the hours just before the dawn venting of symbionts, nearly all of the dozens of host cytoskeletal genes are upregulated, a change that is correlated with the effacement of the epithelial microvilli into the crypt spaces; the ultrastructure of the host's tissues at this point looks remarkably like the effacement of epithelia by enteric pathogens (80). After 6 h, i.e., when most of the symbionts have been vented and the remaining population has begun to grow exponentially, the bacterial symbionts have upregulated genes associated with the metabolism of host-membrane constituents. Specifically, the genes associated with anaerobic respiration of glycerolphosphate are induced, and an analysis of the lipid composition of the bacterial membranes indicates that the symbionts also incorporate host-specific membrane fatty acids into their own membranes. Over this same time interval, the host downregulates the cytoskeletal genes and begins to reconstitute a new microvillous layer on the existing crypt epithelial cells. By around noon, the symbionts have once again filled the crypt spaces, and their growth is curtailed. Then, in the late afternoon and evening, the symbionts shift their metabolism to the fermentation of the polysaccharide chitin. Taken together, the data demonstrate a day-night oscillation of the symbiont's metabolic pathways from anaerobic respiration to fermentation (94). This rhythmic switch in catabolic pathways also suggests that the crypt environment varies from a neutral to an acidic pH over the day-night cycle. Although these data provide evidence for a daily reciprocal dialogue between the partners, numerous questions about this process remain to be addressed, including the following: Do the symbionts or their products induce effacement, and how? Do rhythmic ultrastructural and molecular changes occur in other symbioses, such as in the vertebrate gut?

In addition to the opportunity it offers to explore the rhythms themselves, the squid-vibrio system offers an opportunity to characterize how these rhythms are controlled. The first reported evidence for the occurrence of a bacteria-driven circadian rhythm in animals was provided by a recent study in *E. scolopes* (32). The transcription of genes encoding cryptochromes, proteins that are conserved regulators of animal circadian rhythms, has a daily oscillation in the host light organ.
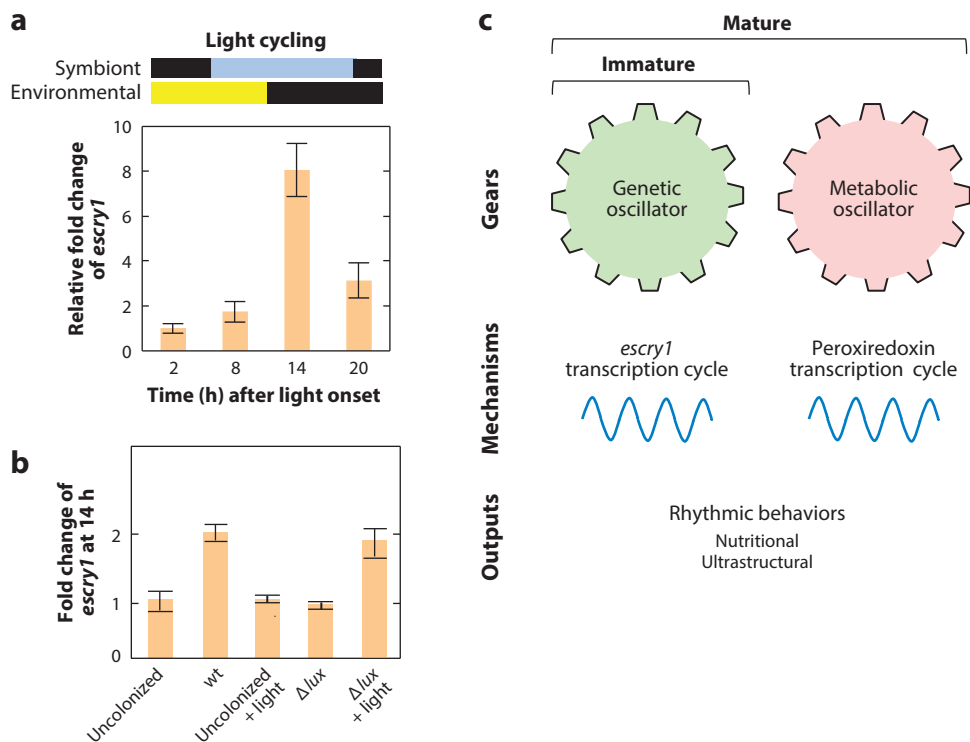
Figure 2

Candidate mechanisms underlying molecular and biochemical control of host-symbiont rhythms. (*a*) *escry1* gene regulation. Analyses of the regulation of one of the two host cryptochrome genes over the day-night cycle (environmental light) revealed that *escry1* gene expression peaks during peak luminescence (symbiont light). (*b*) A mutant defective in light production (Δ*lux*) was unable to induce these symbiosis levels of *escry1* expression. This defect could be complemented by exposing symbiotic animals to external blue light (Δ*lux* + light), but not by exposing uncolonized animals (uncolonized + light) (32). (*c*) The host cryptochrome is one of a family of genetic oscillators; it begins to cycle immediately upon colonization by the symbiont. We hypothesize that daily oscillations in the mature organ are governed by both *escry1*, the genetic oscillator, and peroxiredoxin (PRX), the metabolic oscillator. Whereas in central clocks (the brain), the PRX genes do not cycle, in the peripheral clock (the light organ), expression of these genes does oscillate over the day-night cycle. In addition to studying gene transcription and protein production, one can characterize these behaviors by analyzing metabolic and ultrastructural features. Abbreviation: wt, wild type. Panel *c* modified from Reference 4.

Two cryptochrome genes, *escry1* and *escry2*, have been identified in the host squid. One of these, *escry2*, is dominant in the eye and cycles with environmental light, which is typical of *cry* genes in other animals. However, the transcriptional cycling of *escry1* is offset 12 h and corresponds to the daily rhythm of the symbiont luminescence (**Figure 2a**). Studies with juvenile animals demonstrated that the symbionts are required for the entrainment *escry1* cycling. Further, analyses with a mutant of *V. fischeri* defective in light production (Δ*lux*) demonstrated that symbiont luminescence is also required (32). Whereas the defect in these mutants could be complemented with provision of exogenous blue light, in the absence of symbionts, illumination alone could not entrain the rhythms (**Figure 2b**). These data show that *V. fischeri* must be present to enable the host animal to respond to the daily cycle of its partner's luminescence.

Much remains to be learned about the role and mechanisms of rhythms in this association and about how the early development of these rhythms influences maturation of the symbiosis. Analysis of a newly available RNAseq database has revealed that other key biochemical regulators of rhythms, including *per* and *timeless*, are expressed in the host light organ. Whether the cycling of these genes occurs and, if so, is similar in the eye and the light organ, remains to be determined. We also have evidence that the universal metabolic clock proteins, peroxiredoxins, are expressed in adult *E. scolopes* light organs (94). These redox proteins have many functions, but they may sense the day-night change in symbiont metabolism. Taken together, the data have provided enough information to construct a model of symbiosis rhythms in this system that provides testable hypotheses for future research. For instance, preliminary data suggest that the cycle between anaerobic respiration and fermentation may not be active in the juvenile symbiosis, indicating that a peroxiredoxin-based rhythm may not appear until the organ has fully developed (**Figure 2c**).

## COLONIZATION OF THE HOST LIGHT ORGAN

The embryonic period prepares the animal to interact with environmental *V. fischeri* (See **Supplementary Material** and **Supplemental Figure 2**). At the end of embryogenesis, a complex set of differentiated cell types has developed that will promote harvesting of the symbiont (**Figure 3**). The superficial ciliated epithelium consists of an anterior and posterior appendage, which covers a blood sinus, and a set of cells surrounding the pores (**Figure 3a**). The cells across this field vary in size, length, and ciliary activity (40), but the functional significance of these variations is not well understood. At the time of hatching, what began as invaginations of the embryonic hindgut–ink sac complex has become an elaborate set of blind-ended crypts (60, 85). In the most mature of these (crypt 1), moving lateral to medial, the pore leads to a ciliated duct, which opens into a broader antechamber. At the medial end of the antechamber is a constriction of the tissues, or bottleneck, that opens more medially into a labyrinthine crypt lined by a microvillous epithelium. Although this distance is a total of only ∼100 μm, it covers a series of distinct environments to which the future symbionts must adapt during the colonization process.

To my knowledge, the only horizontally transmitted symbioses in which the initiation of the partnership has been extensively studied are the legume-rhizobia and squid-vibrio associations. Establishment of a specific partnership during initiation of symbiosis (i.e., engagement of the co-evolved partner to the exclusion of other environmental bacteria) is resolved in both systems over

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Supplemental Material

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Figure 3

The symbiont's journey. (*a*) The anatomy of the host organ. The light organ (*circled*) as seen through the ventral surface of an anesthetized hatchling animal. A diagram of the external (*left*) and internal (*right*) features of this organ. (*b, upper*) Colonization events. Early host (*Euprymna scolopes*) engagement of the symbiont (*Vibrio fischeri*) showing events, critical partner interactions, and biomolecules involved, where known. (*b, upper left*) Confocal image of the organ's ciliated field, which is shedding abundant mucus in the regions around the pores in response to the microbe-associated molecular patterns (MAMPs) in environmental seawater (64). (*b, upper middle*) High-magnification confocal image of a living specimen shows symbiont cells attaching to the cilia (2). Experimental manipulations provide evidence that the host's perception of symbiont MAMPs induces both cellular (41) and transcriptomic (43) changes in the host tissues. (*b, upper right*) Colonization in response to a host-generated chitobiose gradient. Following attachment, the symbionts aggregate using an exopolysaccharide (EPS) matrix on their surfaces (90). The observed priming to NO (92) and chitobiose (43) likely occurs in these aggregated cells. Once primed, the *V. fischeri* cells perceive the host gradient and move into the organ. (*b, lower*) With large numbers of green-fluorescent-protein-labeled *V. fischeri* cells, their transit through tissues can be visualized by confocal microscopy (85); host tissues are red. After entering the pores (*step 1*), *V. fischeri* cells move through ducts (*step 2*) lined by outward-beating cilia (*bottom*, transmission electron micrograph), which requires that the symbionts move countercurrent. The symbionts then proceed across the antechamber to a bottleneck (*step 3*). Once in the crypts, symbiont cells proliferate to fill each crypt by ∼12 h postinoculation (*step 4*) (96). Abbreviation: PGN, peptidoglycan.

the first minutes to hours. In contrast, whereas nitrogenase activity characteristic of a functional root nodule takes days to weeks to develop (89), the juvenile squid has a fully colonized, biolumines-cent organ within hours of hatching (76). Nevertheless, in both these and many other horizontally transmitted symbioses, the initiation of an association represents a dramatic niche change for the symbionts, from a diverse, diffuse microbial community to a densely packed, monospecific population.
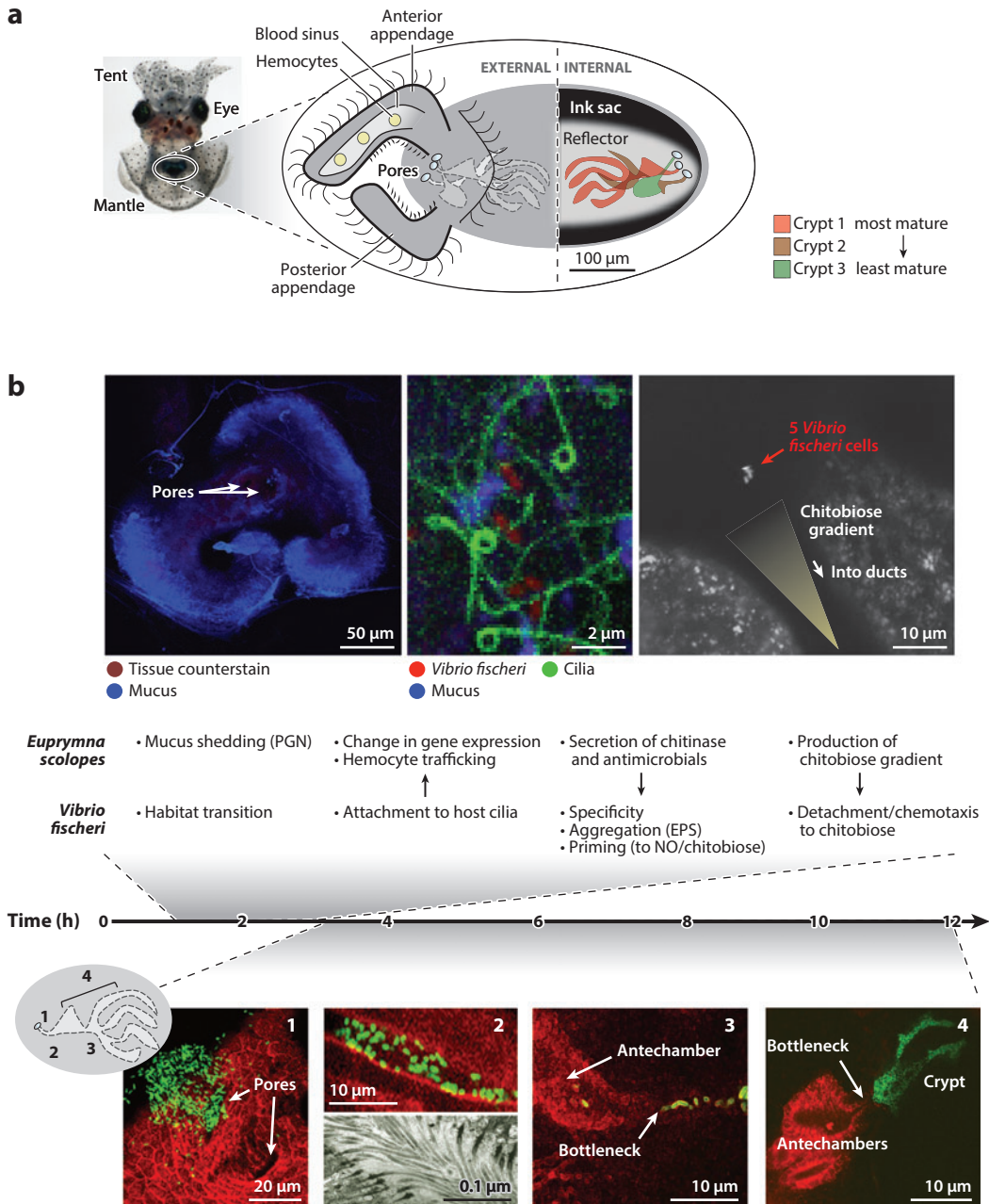
How does this niche transition progress? The initiation process proceeds through a series of stages that includes host-symbiont interactions both at the ciliated surfaces and deep within the tissues (**Figure 3b**). Within seconds of hatching, the animal begins to ventilate environmental seawater through its body cavity. The host accomplishes two interrelated processes during the subsequent 3- to 4-h period: It harvests its *V. fischeri* inoculum from the seawater and excludes all other bacterial species from successful engagement. This harvesting is an impressive feat considering that, in near-shore Hawaiian seawater, *V. fischeri* typically occur at no more than a few hundred to thousands within communities of millions of other bacteria per milliliter (36, 46). The initiation events, which begin along the ciliated epithelia, can be observed directly by confocal microscopy. The *V. fischeri* cells first attach to the cilia on the epithelial surface and then form small aggregations of 5–10 cells when the bacteria are present at a typical seawater concentration (2). Although under the experimental conditions of the laboratory aggregates of hundreds of cells can be obtained with large inocula (66), changing the inoculum size does not influence the timing of colonization. After 3–4 h, the aggregated cells undergo a tortuous migration into deep tissues (**Figure 3b**) (85). On the last leg of this journey, the symbionts proceed single file through bottlenecks just outside of the crypts. Even with a large inoculum, apparently only a few inoculating *V. fischeri* cells will proliferate to fill any given crypt (96). Remarkably, even in the absence of *V. fischeri*, although other species initially associate with the epithelial surface, none will colonize the crypts.

Several studies have investigated the biochemical underpinnings of this process. The few hours of residence as an aggregate on the organ surface not only select for the specific symbiont but also prepare them for the journey. Shortly after hatching, in response to the peptidoglycan (PGN) present in natural seawater, the epithelial fields shed copious mucus (**Figure 3b**), which may serve both biophysical and biochemical functions during initiation. As mentioned above, the mucus contains antimicrobial molecules, including nitric oxide (NO) that is produced by host NO synthases (NOS) in the ciliated epithelia and transported into the mucus in vesicles (13). Although this NO could discourage aggregation by some bacterial species, its main role appears to be in priming *V. fischeri* cells to resist their subsequent encounter with more intense stresses (92). Specifically, whereas NO first appears in the mucus, it is in the duct and antechambers that NOS/NO are most abundant (13, 85). Genetic studies of *V. fischeri* have determined that induction of NO resistance during the attachment and/or aggregation stages is essential for completion of colonization (93).

Association of *V. fischeri* cells with the cilia (**Figure 3b**) induces hemocyte trafficking into the blood sinuses of the organ. Although the precise function of this cell migration is not known, these data demonstrate that the host detects the presence of as few as 4–5 cells and responds with a robust cellular phenotype (43). Host hemocyte trafficking results from the sensing of symbiont PGN monomer, or tracheal cytotoxin (TCT), which is constitutively exported by *V. fischeri* cells (41). The response to so few cells attests to the highly specific activity of the TCT molecule. Sensitivity to the few attaching *V. fischeri* cells, against a background of other, nonspecific bacterial cells in the ambient seawater, also results in a robust change in host gene expression. Upregulated genes include several that encode antimicrobial proteins and those that encode peptidases and a chitotriosidase. From the symbiont's side, the bacterium-bacterium aggregation that follows ciliary attachment requires the release of a constitutively produced exopolysaccharide (EPS) (49, 97). The genetic control of the pathway responsible for EPS release has been extensively studied (61, 90). Nevertheless, the function of EPS-mediated aggregation has not been determined, although it may be essential for critical cell-cell communication, detachment from the cilia, or protection from the harsh environment of host tissues. Interestingly symbionts defective in the production of a secreted aminopeptidase-N aggregated poorly and colonized slowly (23), perhaps because the peptidase activity is critical for interaction with either the cilia (attachment or detachment) or adjacent *V. fischeri* cells prior to migration.

Aggregated *V. fischeri* cells are poised to enter the nascent light organ (**Figure 3b**) but require a number of additional traits to complete colonization, including flagellar motility and chemotaxis (8, 15, 27). Remarkably, the upregulation of the host chitotriosidase mentioned above (43) plays a critical role in this migration. Chitotriosidase is endochitolytic, cleaving chitin polymers into dimeric chitobiose, and *V. fischeri* moves into the pores on the light organ surface in response to a chitobiose gradient (48). Culture studies of *V. fischeri* demonstrated that they require priming by chitobiose prior to migrating toward this molecule. Because the host chitotriosidase is secreted into the host's mucus, which contains chitin, it catalyzes the production of chitobiose that primes the aggregated *V. fischeri* cells (43). A more concentrated chitotriosidase activity in the ducts apparently produces the gradient to which *V. fischeri* responds as it enters the light-organ pores (43, 48).

As with the legume-rhizobium symbiosis, initial interactions between *E. scolopes* and *V. fischeri* require a complex host-symbiont dialogue. Clearly, many questions remain unanswered, including some that must await further tool development. Application of bacterial molecular genetics has been a powerful approach revealing critical symbiont features (75); however, genome-wide transcriptomic analyses of the small number of aggregating *V. fischeri* cells have not yet been possible. Future development of single-bacterial-cell transcriptomics will provide a window into the genetic regulation involved in a symbiont's physiological transition from the planktonic to the symbiotic state. Conversely, whereas transcriptomic approaches are possible in the host (11, 43), development of methods for genetic knockdown of host genes, although ongoing, is not yet complete. Together, these advances should provide insight into why *V. fischeri* is the only bacterium that can leave its seawater habitat and colonize the *E. scolopes* light organ.

SYMBIONT TRIGGERING OF LIGHT-ORGAN MORPHOGENESIS: LOCATION, LOCATION, LOCATION!

All major light-organ regions through which *V. fischeri* cells pass during colonization (**Figure 3b**) undergo symbiont-induced developmental changes. These modifications in morphology and function appear to transform the organ from a form that promotes colonization to one that fosters a life-long relationship with the symbiont. Once *V. fischeri* cells have populated the crypt spaces, their presence induces alterations in the tissues, only some of which can be reversed by antibiotic treatments that eliminate the symbionts (17). The most dramatic of the changes is the loss of the superficial ciliated epithelium (**Figure 4a**). This process, which results from apoptosis of the host cells, takes ~4–5 days but has already been irreversibly triggered by ~12 h after inoculation (17), at which time the symbiont population has colonized the crypts (76). Although some symbiont activities that trigger this process have been defined, how they signal the apoptotic events from the crypts, located several cell layers away, is not known. As such, this process is reminiscent of the triggering of cortical cell division in the roots of legumes in response to rhizobia interacting with the root hairs (68). In the juvenile squid, apoptosis of the superficial epithelial layer is accompanied by breakdown of the supporting basement membrane through the activity of matrix metalloproteinases (40). Cells detach from the basement membrane into the mantle cavity, and the cytoskeleton of the surrounding cells coordinately mobilizes to zip closed the space created by the leaving cell. In addition, within 24–48 h of colonization, the symbionts shut down shedding of mucus from the surface epithelium (64). Unlike the apoptotic morphogenesis, this developmental change is reversible; if the crypts are cured of the symbionts, the surface cells once again initiate mucus shedding, and symbionts will again begin to aggregate, although at lower numbers than previously (64).

The interior tissues undergo more subtle changes, which are most often reversible. Although *V. fischeri* cells migrate through the ducts and antechambers of the organ, they do not reside there; i.e., they interact with these tissues only during the initial colonization event and as they
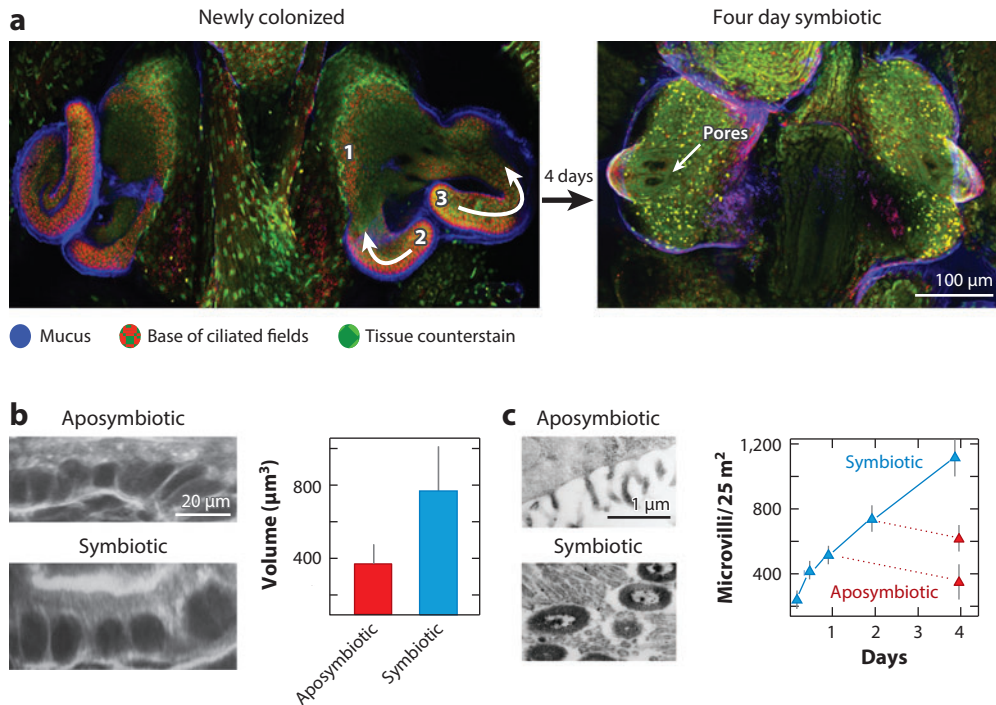
a Newly colonized — Four day symbiotic

● Mucus ● Base of ciliated fields ● Tissue counterstain

b Aposymbiotic / Symbiotic

c Aposymbiotic / Symbiotic

Figure 4

Symbiont-induced light-organ morphogenesis that follows crypt colonization. (*a*) The four-day program of regression of the ciliated epithelial fields on the organ surface occurs through apoptosis, which is induced by symbiont populations deep within the crypts. (*a, left*) The process begins with the loss of the ridge of ciliated cells just medial to the pores (*step 1*), followed by shortening and loss of the posterior (*step 2*) and anterior (*step 3*) appendages. (*b,c*) Changes of the crypt epithelia that result from direct interactions between host and symbiont cells, swelling of crypt epithelial cells (*b*), and increase in the density of the microvilli on the surfaces (*c*). Eliminating *Vibrio fischeri* cells at 1 or 2 days with antibiotic treatment (*dashed lines* in graph, panel *c*) causes a return to the aposymbiotic morphological state of this tissue.

are released during the daily expulsions (85). The timing of developmental changes in these tissues in response to colonization suggests that the effects of *V. fischeri* are also not the result of direct host cell/bacterial cell interactions. By 12 h, the ducts have constricted ∼50%, (i.e., from ∼30 μm down to 15 μm) because of a decrease in the number of cells surrounding the duct lumen (37). This reversible change is correlated with an increase in actin synthesis and abundance of filamentous actin in the terminal web of the duct cells. By ∼7 days, the three pores on either side of the organ (**Figure 3a**) begin to coalesce into a single orifice, characteristic of the adult condition (**Figure 1b**). Similarly, by 48 h the bottleneck narrows from ∼9 μm to ∼2 μm (85), slightly wider than a single *V. fischeri* cell. The underlying mechanisms of bottleneck constriction, and whether this event is reversible, have not been explored. In contrast, direct interaction with *V. fischeri* cells induces modifications of the crypt epithelia with which the symbionts associate, and these changes can also be reversed by curing. Specifically, by 48 h following colonization, the symbiont population has induced an approximately twofold increase in cytoplasmic volume (58, 85, 91) and an approximately fourfold increase in the density of the microvilli (44) of the crypt epithelial cells (**Figure 4b,c**). These modifications result in a greater intimacy between host cells and symbionts.

Numerous studies have investigated symbiont features that induce these morphological and ultrastructural changes, and these are the subject of recent reviews (53, 55); thus, this topic is not

covered in detail here. However, the surprising finding was that conserved MAMPs, a term that was first used in a publication on development of squid-vibrio symbiosis (41), are key inducers of symbiont-mediated host development. Specifically, lipopolysaccharide (LPS) and PGN derivatives are potent morphogens and work synergistically to induce much of the regression of the superficial epithelium (1, 41, 87). Their likely involvement in other developmental events occurring within internal host tissues is the subject of ongoing studies. Interestingly, these MAMPs, similar to their role in pathogenesis, appear to modulate immune responses. For example, beyond triggering apoptosis, they induce hemocyte trafficking into the light organ (41). In addition, LPS and PGN derivatives work synergistically to attenuate NOS activity and NO production (3), which is the opposite of how these molecules function in pathogenesis, where MAMPs typically increase nitrosylative stress (see, e.g., 24, 38).

Evidence is accumulating that light production by the symbiont synergizes with these chemical MAMPs, and together they drive much, if not all, of the developmental program (53). Although the basis of light perception by host tissues is not well understood, two possible mechanisms have been discovered. The above-mentioned cryptochromes, which are blue light receptors, may perceive and respond to the blue luminescence of the symbiont. Cryptochromes have also recently been implicated in induction of plant development (47). The second possible receptor is rhodopsin. In addition to having much the same structure as the eye (86), the light organ produces all of the protein components of the eye's visual transduction cascade, including the visual pigment rhodopsin, and exhibits physiological sensitivity to light, much like the eye (86). Whatever the receptor, the mechanism of action is still unknown. *V. fischeri* mutants defective in light production are also defective in normal transcription of the light organ's eye-specification genes, which may be involved in developmental induction by luminescence (71). Further, a microarray study demonstrated that bacteria defective in luminescence are not capable of inducing normal transcriptional levels of conserved host pattern-recognition receptors (11), which may be the receptors for the MAMPs that are critical for inducing normal development. Finally, transcription of key genes of the NF-κB pathway occurs in the light organ. As the principal response mechanism to MAMPs, this pathway presents a likely link between symbiont signals and host responses. Obviously these various jigsaw pieces, while suggesting an underlying image, do not yet provide a coherent picture. Much research will be required to fully understand how light and MAMPs function together to drive developmental processes.

FRONTIERS

New horizons in the *E. scolopes–V. fischeri* symbiosis are strongly linked to the ongoing development of tools for dissecting and revealing the events underlying the initiation and maintenance of the system. Three emerging technologies deserve mention here. For much of the first 25 years that the squid-vibrio symbiosis has been studied, research has focused on the association's first few days. While this period of onset and early development offers a rich landscape for experimentation, it had been delimited by the technical difficulty in raising the juvenile host beyond about one week (31). With intensive efforts over the last few years, robust methods for raising the animal to maturity have been developed. Our initial experiments with this new capability indicate that the association is surprisingly sensitive to the presence of nonluminous *V. fischeri* and over the course of four weeks is able to essentially clear a population of such cheaters, even when cocolonized with a normal luminous symbiont (39). Whereas uncolonized animals continue to be susceptible to symbiotic infection for at least several weeks after hatching, colonization triggers a critical change in the symbiosis' subsequent receptivity and resilience (39). Specifically, animals colonized for three days can be cured and successfully recolonized; however, after five days they cannot. The

capability to experimentally manipulate the host through all stages of ontogeny opens a whole new arena for research into the squid-vibrio symbiosis.

The continuing development of new molecular genetic technology has opened exciting directions for study of both host and symbiont. For example, sequencing of the genomes of dozens of *V. fischeri* strains, both symbiotic and strictly environmental, is underway. Comparative analysis of the resulting data has begun to provide insight into that portion of the flexible genome of *V. fischeri* that is required for the establishment and maintenance of a horizontally transmitted animal symbiosis (81, 95). The sequencing of the host genome is nearly complete and will pave the way for the study of such exciting questions as the nature of gene regulation during the developmental trajectory of the association. In addition, attempts at genetic manipulation of the host are continuing, and refinement of the methods for the transcriptomic, proteomic, and metabolomic analyses of small amounts of tissue will provide an opportunity to unravel the temporal and spatial details of symbiosis development. The ultimate goal is to understand the complex dialogue between symbiotic partners from a biochemical to an ecological level.

Finally, the squid-vibrio association also offers rare opportunities for merging the fields of biophysics and mathematics into the investigation of symbiosis, an exciting new synergy that is currently under development. For example, the initial interaction between *E. scolopes* and *V. fischeri* offers the rare chance to visualize and model the capture and selection of bacterial cells along ciliated surfaces in an intact animal. The vision is that a thorough understanding of this process will inform many areas of biomedical research about how animals establish healthy interactions along their ciliated epithelial surfaces while dissuading pathogenic ones.

In conclusion, the squid-vibrio system is just one of a number of genetic models of animal-bacterial symbiosis that have been pioneered over the last few years (7, 42, 57, 75). Evolution has done remarkable experiments with this phenomenon, providing biologists with a rich palette for research. Just as models have been invaluable for deciphering how animal development programs themselves are executed, together these symbiosis models, and those to be generated in the future, promise to provide a coherent picture of both evolutionarily conserved features and the mechanisms driving diversification that underlie the development of host-microbe interactions.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

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