Supplemental Materials

THE PHYLOGENETIC POSITION OF THE PARTNERS AND PATTERNS OF THE SYMBIOSIS

The phylogenetic position of the squid host (**Figure S1, upper left**) places it in an understudied group of animals, making the discovery of the molecular underpinnings of symbiosis development challenging. Specifically, the host resides in a major division of the animal kingdom, the Superphylum Lophotrochozoa, which includes mollusks, e.g., squids (cephalopods), snails, clams, and their relatives. Far fewer 'omics' data are available for the lophotrochozoans relative to the more widely studied Ecdysozoa, home taxon for the fruit fly and worms, and Deuterostomia, which includes the vertebrates. In fact, when it was published in 2008, the juvenile *E. scolopes* light-organ EST database (3), with a UniGene set of ~14,000, became the largest set of transcripts for any lophotrochozoan. This database has been growing exponentially, and sequencing of the *E. scolopes* genome, which is about 107% the size of the human genome, is now underway. Thus, with the appearance of these new tools, the prospect is bright for continued progress in discovering the molecular roots of symbiosis development.

Several squid species have bacterial light organs (**Figure S1, lower left**). Recent molecular phylogenetic analyses of this trait's patterns of occurrence provide evidence that bacterial light organs arose independently in the two major squid groups in which they occur, the loliginids (arrow squids) and the sepiolids (the bobtail squids) (19). Interestingly, however, the data also suggest that within the sepiolids, which have several genera with bacterial light organs, such organs may have been lost in one genus, *Sepietta*.

The gram-negative luminous symbiont of *E. scolopes*, *V. fischeri,* is classified within the family Vibrionaceae of the Vibrionales, which are γ-proteobacteria, a clade that has many species forming symbioses with animals. (**Figure S1, upper right**). Most, if not all, squids in the genus *Euprymna*, which is confined to the IndoPacific, harbor only *V. fischeri* strains in their light organs (28). In contrast, the Mediterranean and Atlantic sepiolids in the genus *Sepiola* can co-harbor *V. fischeri* and *V. logei*, a close relative of *V. fischeri* (9; 46) (**Figure S1, lower right**). The genus name for these two bacterial species has been changed several times, but a recent analysis of full-genome sequence data for members of the Vibrionaceae now support placement of *V. fischeri* and *V. logei* in the genus *Vibrio* (6).

V. fischeri has a broad geographic range and, due to its physiological versatility (8), occurs in a variety of niches in temperate and subtropical regions. In addition to light-organ symbioses with squids and fishes, it can be found in the bacterioplankton as gut symbionts, as saprophytes, and in sediments (for review, see (27). However, as in other *Vibrio* spp. (22; 35), a given strain is likely to have a principal niche that is relatively narrow. For example, studies of the *E. scolopes*-*V. fischeri* association demonstrate that, not only is symbiosis the defining niche of strains occurring in the squid host, but also these strains are more fit in particular partnerships. For instance, in the bacterioplankton of Hawaiian coastal seawater, the concentration of *V. fischeri* cells declines dramatically beyond a kilometer from an abundant *E. scolopes* population (18), suggesting that, while the bacterium survives well in seawater (44), its biogeography is essentially limited by the distribution of the symbiosis. Even on the island of Oahu, Hawaii, subpopulations of the squid and its symbiont show apparent co-divergence. For example, both the host and the bacterium collected from bays on the northern or southern sides of Oahu are genetically distinguishable (16; 44), and a juvenile from one side of the island is not colonized

by *V. fischeri* strains from the other side as well as those from their own habitat (44). Interestingly, experimental serial passage through successive Hawaiian squid hosts has been reported to render a non-native strain more fit in the symbiosis, and phenotypically more like the natural symbiont (34). The genetic basis for symbiotic fitness differences between strains of *V. fischeri* is becoming clearer. In a recent benchmark study of this phenomenon, a comparative genomic approach was performed to identify squid-symbiont genes that are missing in a *V. fischeri* strain that was isolated from the light organ of a fish species, but is a poor colonizer of the *E. scolopes* light organ (21). This screen yielded one such squid symbiont-specific gene, *rscS*, which encodes a regulator of exopolysaccharide production (45) that plays an important role in initiating colonization. Introduction of an *rscS* allele into the fish symbiont rendered it capable of efficient colonization of the squid, thus identifying it as the first example of a bacterial regulatory gene responsible for a function that mediates host specificity (21).

THE MORPHOLOGY OF THE LIGHT ORGAN: THE EYE-LIKE FEATURES OF THE LIGHT ORGAN, AND ITS ASSOCIATION WITH THE SQUID INK SAC

Bacterial light organs are anatomically complex and, from their morphology (39), to their biochemistry (4; 24; 39), show striking similarities to image-forming eyes. Thus, they offer a compelling example of evolutionary tinkering (13), i.e., the coopting of preexisting elements for a new function. The similarities between the eye and light organ were underscored by a recent comparison of these structures through embryogenesis. The data revealed that the eye-specification genes, including the master regulatory gene *pax6*, are expressed in the light organ during development (32), which suggests that eyes and light organs form in response to the same program of gene regulation. In addition, like the eye, light organs are energetically demanding to

develop and maintain. As such, should the animal's ecology change, e.g., from nocturnal to diurnal activity, the bacterial light organ would lose its fitness value, and selection on developmental programs might drive their rapid loss. An analogous selection has been extensively studied in the evolutionary loss of eyes of the blind cavefish (14), a process that occurred over only a few million years (33) and that largely involved a change in regulation of the *pax6* gene (36). Whether a change similar genetic regulation of *pax6* by change controlled the loss of light organs in *Sepietta* spp. (**Figure S1, lower left**) remains to be determined.

Interestingly, the bacterial light organs of squids always develop in association with the ink sac, the diverticula of which provide one mechanism whereby the animal controls light emission from the organ (23; 26). In sepiolid species with light organs, the ink sac develops in a more anterior position, i.e., in those members of this family without light organs that have been examined, the ink sac is further toward the hind end of the animal (10). Light organs may also develop in association with the ink sac because of its biochemistry; proteins associated with antimicrobial function, notably nitric oxide synthase and halide peroxidase, are abundant in ink sac epithelia, and are critical for ink production (31). Significantly, the activity of these two enzymes is regulated by *V. fischeri* in the squid light organ (5; 42). While a microbial symbiosis developing in a region with strong antimicrobial activity may seem counterintuitive, it is not unusual. Approximately 11% of all insects have intracellular bacterial symbioses that are associated with the fat bodies, the major site of antimicrobial peptide production (7), and recent evidence suggests that the activity of these peptides is essential for balancing the symbiotic state in mutualistic associations (20).

A BRIEF PERIOD WITHOUT THE SYMBIOTIC PARTNER – EMBRYOGENESIS PREPARES THE WAY

Although symbionts do not directly interact with embryonic tissues during the development of horizontally transmitted symbioses, embryogenesis must prepare the nascent host for its eventual interactions with microbial partners. For example, bacterial fermentation in vertebrates can occur in the foregut, caecum and hindgut (15) and, in each case, rudimentary tissues develop during the embryonic period, the form and function of which poise the tissues to establish healthy alliances with their coevolved partners (see e.g., (1; 2; 11). In an analogous manner, embryogenesis in the squid host, as detailed below, produces a conspicuous set of tissues ready for symbiont acquisition.

The adult *E. scolopes* female lays, on average, clutches of ~150 eggs (**Figure S2*a***) and will produce ~15 clutches over a 4-mo reproductive period. Maintaining an active breeding colony of 8 productive females and 8 males yields ~60,000 juveniles/yr. The embryonic period of *E. scolopes* typically occurs over 18-25 d, depending on environmental conditions (e.g., temperature and aeration level). During that time, morphological and biochemical determinants are developed that facilitate colonization by *V. fischeri* cells upon the egg's hatching (5; 17; 25; 41). Cells along the lateral surface of the developing ink sac proliferate to produce populations that give rise to the features of the hatchling light organ (**Figure S2*b***). The nascent crypts form as invaginations in these regions (**Figure S2*c***). The three crypts on each side of the organ, which we designate crypts 1, 2, and 3, form sequentially, beginning with the crypt 1 about half way through embryogenesis; the invagination to form crypt 3, which is last to develop, begins only a few days before hatching. This sequence of development results in variation in the crypts' relative maturity at the time of hatching. Outgrowths of the lateral

surfaces of the embryonic rudiment form concomitantly with the crypts (**Figure 2S*d***), ultimately producing the juvenile-specific epithelial fields that function to harvest symbionts from the bacterioplankton. Both the surface epithelium and the immature crypts are densely ciliated. In the newly hatched juvenile the deep portions of crypts 1 and 2 have lost the cilia along their epithelial surfaces and matured to the microvillous condition that persists throughout the life of the animal. On occasion, the apical surfaces of crypt 3 epithelia are still ciliated at hatching, which is likely why this crypt often responds differently to interactions with *V. fischeri* cells (38). Although the biochemical adaptations of the hatchling crypt environment that will mediate a successful colonization have not been well characterized, the superficial ciliated fields involved in harvesting the symbiont have received considerable attention. In addition to being readied for mucus secretion (29; 30), which occurs shortly after hatching, embryogenesis also provides the hatchling's ciliated field with abundant stores of antimicrobial compounds, including nitric oxide synthase (5) and a peptidoglycan recognition protein (PGRP) (40).

Supplementary figure legends

Figure S1. Cladograms illustrating the relationships of the host (12; 37) and the symbiont (6; 43) within their respective domains. *, clades with symbiotic systems where well-developed host genetics are available; †, examples of the clades of host and symbiont with members that develop light organ symbioses; such associations are thought to have evolved independently in the squids several times (19). *V. fischeri* image (lower right) provided by Dennis Kunkel Microscopy, Inc.

Figure S2. Embryogenesis, the developmental period that occurs in the absence of the symbiont. *(a)* An adult female surveying a clutch of ~200 eggs, which she has laid on hard substrate provided in the culture system. *(b)* A ventral view of an embryo removed from the egg capsule.

This image of an individual about half way through embryogenesis shows the light organ (white square) developing as a rudiment associated with the ink sac. *(c, d)* Light organ about two-thirds of the way through embryogenesis. *(c)* A surface view reveals the developing anterior appendage, and early invaginations at the pores. *(d)* A deeper view shows a duct, medial to one of the pores.

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Figure S1

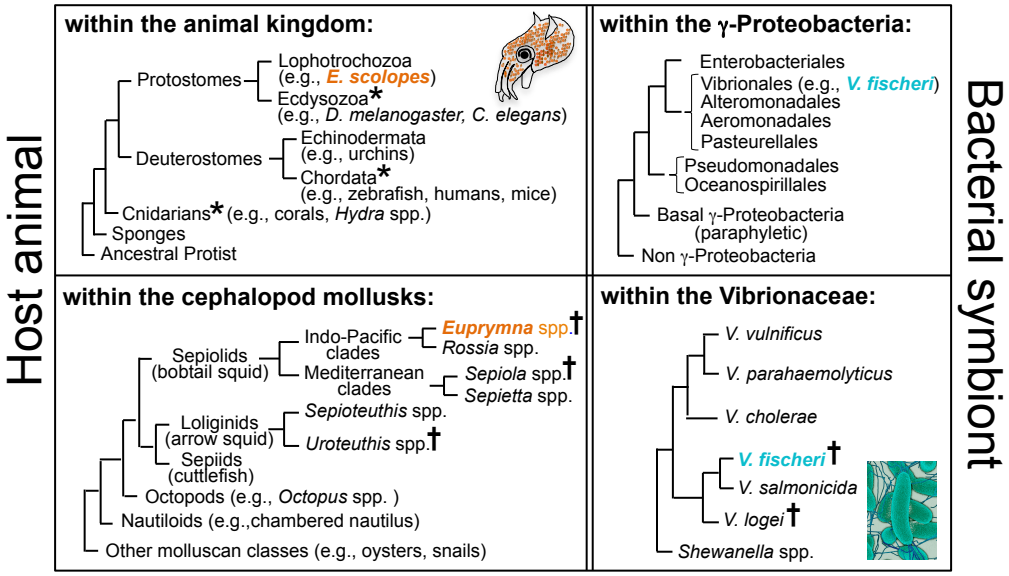

Figure S1
Cladograms illustrating the relationships of the host (33; 108) and the symbiont (19; 121) within their respective domains. *, clades with symbiotic systems where well-developed host genetics are available; †, examples of the clades of host and symbiont with members that develop light organ symbioses; such associations are thought to have evolved independently in the squids several times (60). *V. fischeri* image (lower right) provided by Dennis Kunkel Microscopy, Inc.

Figure S2

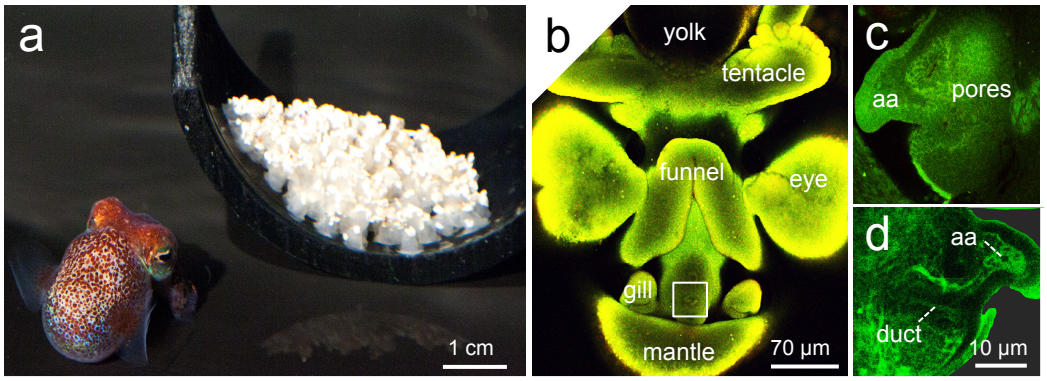

Figure S2

Embryogenesis, the developmental period that occurs in the absence of the symbiont. *(a)* An adult female surveying a clutch of ~200 eggs, which she has laid on hard substrate provided in the culture system. *(b)* A ventral view of an embryo removed from the egg capsule. This image of an individual about half way through embryogenesis shows the light organ (white square) developing as a rudiment associated with the ink sac. *(c, d)* Light organ about two-thirds of the way through embryogenesis. *(c)* A surface view reveals the developing anterior appendage, and early invaginations at the pores. *(d)* A deeper view shows a duct, medial to one of the pores.