A lasting symbiosis: how the Hawaiian bobtail squid finds and keeps its bioluminescent bacterial partner

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Abstract | For more than 30 years, the association between the Hawaiian bobtail squid, *Euprymna scolopes*, and the bioluminescent bacterium *Vibrio fischeri* has been studied as a model system for understanding the colonization of animal epithelia by symbiotic bacteria. The squid–vibrio light-organ system provides the exquisite resolution only possible with the study of a binary partnership. The impact of this relationship on the partners' biology has been broadly characterized, including their ecology and evolutionary biology as well as the underlying molecular mechanisms of symbiotic dynamics. Much has been learned about the factors that foster initial light-organ colonization, and more recently about the maturation and long-term maintenance of the association. This Review synthesizes the results of recent research on the light-organ association and also describes the development of new horizons for *E. scolopes* as a model organism that promises to inform biology and biomedicine about the basic nature of host–microorganism interactions.

Mutualism
The fitness of both symbiotic partners is enhanced by the association.

Parasitism
(Or pathogenesis). The fitness of one partner is enhanced and the other is diminished.

Commensalism
The fitness of one symbiotic partner is enhanced and the other is unaffected.

Microbiota
Often refers to the group of microorganisms found in a specific habitat, such as a host or biofilm.

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In the nineteenth century naturalist Anton de Bary coined the term symbiosis and defined this phenomenon as a specific and stable relationship between two different species regardless of the impact of the association on partner fitness (for example, mutualism, parasitism (or pathogenesis) and commensalism)[1]. The state of symbiosis is therefore context dependent, and there is a growing trend to avoid categorizing microbial species based on terms such as pathogens, mutualists or commensals[2]. Animal and plant hosts often form symbioses with microbial partners, such as bacteria, archaea, eukaryotic microorganisms or any combination of these clades. The field of symbiosis has undergone a renaissance in recent years, and a growing number of biologists and biomedical researchers recognize that the microbiota and microbiome of animal hosts, which are widespread in nature, fundamentally influence host health and disease, including development, immune system function, metabolism and even behaviour[3]. Advances in this field have been enabled by novel technologies, including new methods of nucleic acid sequencing, refined imaging and the development of multi-omics approaches. Coupled with these tools, the development of powerful experimental model systems is transforming our understanding of the form and function of symbiotic systems[4,5].

Model associations offer the opportunity to manipulate the partnerships experimentally to reveal the mechanisms underlying their establishment and maintenance[4,5]. One such symbiosis is between the Hawaiian bobtail squid, *Euprymna scolopes*, and the marine bacterium *Vibrio fischeri*, which produces light that the host uses as camouflage in a nocturnal behaviour called counterillumination that disrupts the host's silhouette[6,7]. This association, which has been studied intensively since 1989, provides insights into how symbiotic bacteria influence all aspects of host biology, including evolution and ecology as well as the cellular, biochemical and molecular features of the partners that promote the functioning as a holobiont (also known as metaorganism). The research community that studies *E. scolopes* symbioses embraces the opportunity to work with the natural genetic variation presented by wild-caught animals. Individual egg clutches typically have hundreds of eggs, from which subsets of juveniles hatch simultaneously at dusk. Thus, a large number of replicates within a treatment provide statistical power to experiments, and the inherent genetic variation within and between mated pairs and egg clutches can be characterized to define those features that are conserved across the existing natural variation of the host. In essence, what is conserved across the genetic landscape is likely to be crucial for the form and function of the symbiosis.

The squid–vibrio partnership is highly specific, with only *V. fischeri* colonizing a specialized light organ, which develops from the rudiment of the embryonic hind gut–ink sac complex of the host[8–10] (FIG. 1). The symbiosis is established in each host generation via colonization by environmental cells of *V. fischeri*, which make

Microbiome
Often used interchangeably with the term microbiota. However, some researchers use microbiome to refer to the collective genomes of the microbiota.

up less than 0.1% of the bacterioplankton in the host habitats[11]. Several host- and symbiont-mediated mechanisms ensure specificity (FIG. 2), and in the absence of *V. fischeri* cells in the environment, the light organ remains uncolonized[6]. A hallmark of the squid–vibrio symbiosis has been the ability to characterize experimentally the colonization process over the first hours to days after the hatching of the host, as well as the ability to manipulate *V. fischeri* genetically, which has provided insights into influences on the host that specifically result from genetic modification of the symbiont.

In 2004, 15 years after the first work on the light-organ system, we reviewed the state of knowledge of the initial colonization in the squid–vibrio association[12].

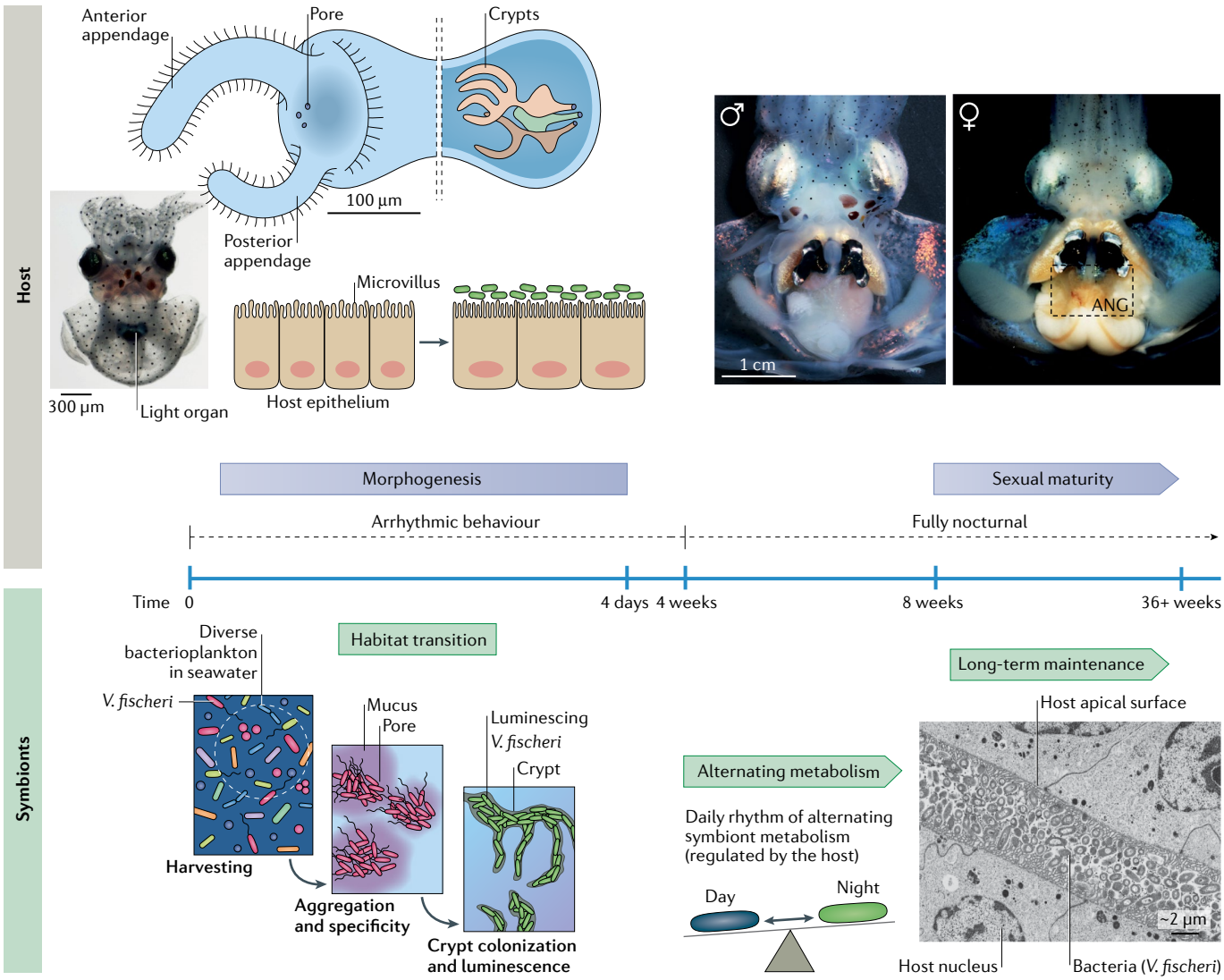


Fig. 1 | **The Hawaiian bobtail squid as a model host for studying symbiosis.** The light-organ symbiosis in *Euprymna scolopes* involves a series of developmental events that ensure successful colonization by *Vibrio fischeri* and the long-term maintenance of the association. At hatching, juvenile squid (upper left) have a nascent light organ with a superficial ciliated epithelium containing appendages that help to recruit environmental *V. fischeri*. Three pores on either side of the light organ lead to epithelium-lined crypts of distinct sizes (crypt 1 in peach, crypt 2 in brown and crypt 3 in green). Colonization occurs within hours after hatching as *V. fischeri* undergoes habitat transition from a free-living bacterium found in the bacterioplankton to aggregating in host mucus, the first site of specificity (lower left). Aggregation is followed by migration through the pores and colonization of the crypts where bioluminescence is induced (lower panel left; an inoculum larger than normal was used to visualize the aggregating cells in relation to the host tissues). Colonization leads to light-organ morphogenesis over the first 4 days of the association. Cellular changes include epithelial swelling and an increase in microvillar density (upper panel left) such that the crypt spaces of fully colonized hosts contain a dense population of *V. fischeri* in direct contact with host epithelial cells (lower right). Over a period of approximately 4 weeks, the light-organ symbiosis matures, and host behaviour changes from being arrhythmic to becoming fully nocturnal. A diel rhythm of alternating symbiont metabolism is also established, during which the symbiont switches between glycerol phosphate respiration and chitin fermentation in the day and night, respectively (lower right). This alternating metabolism helps to facilitate luminescence (that is, the light organ becomes acidic at night, which increases the availability of oxygen used in light production). Sexual dimorphism is evident after *E. scolopes* reaches maturity at approximately 8 weeks after hatching. Female squid have a second symbiotic organ, the accessory nidamental gland (ANG), that houses a simple bacterial consortium (upper right; see BOX 3). Juvenile squid adapted with permission from REF.[14], Annual Reviews. Male squid image courtesy of William Omerod, copyright M. McFall-Ngai. Female squid reprinted with permission from REF.[66], University of Chicago Press. Transmission electron micrograph courtesy of Mary Montgomery, copyright M. McFall-Ngai.

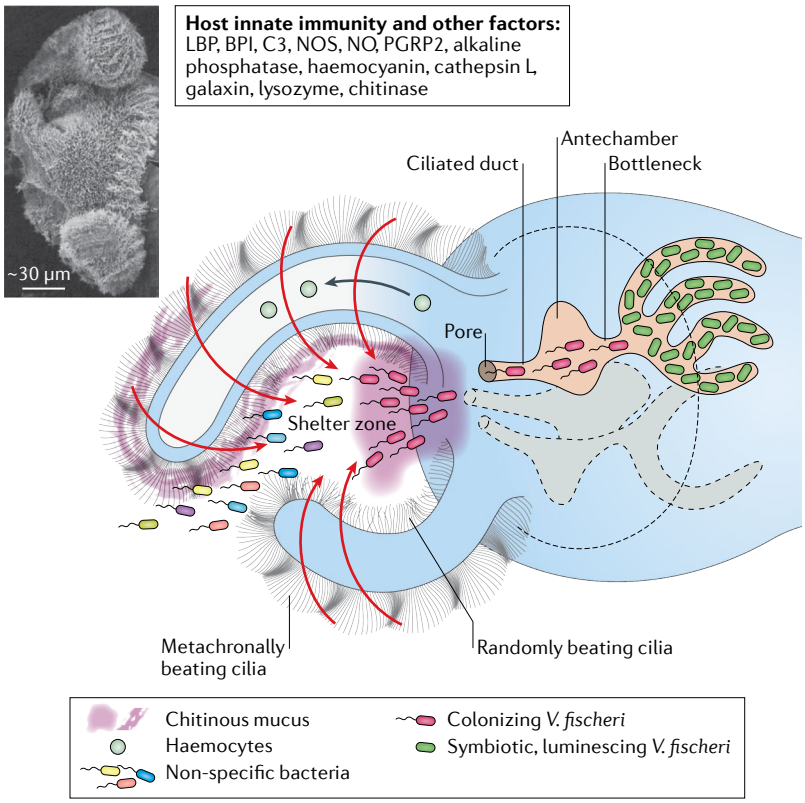


Fig. 2 | **Update to the winnowing model of colonization.** Initial colonization of the light organ involves several biochemical and biomechanical mechanisms to establish the partnership between the Hawaiian bobtail squid, *Euprymna scolopes*, and the bioluminescent bacterium *Vibrio fischeri*. The habitat transition of *V. fischeri* from bacterioplankton to light-organ symbiont involves several features that help to facilitate colonization. The nascent light organ consists of ciliated fields (inset: half of a light organ as shown by a scanning electron micrograph) that are composed of metachronally and randomly beating cilia that create microcurrents (indicated by the arrows) that help to focus bacterium-sized particles above three pores on either side of the light organ in a shelter zone. The host increases expression of endochitinase and secretes mucus that contains several biochemical factors including chitobiose, a symbiont chemoattractant, and a suite of host immunity factors that may serve to inhibit other bacteria. This unique microenvironment selects for *V. fischeri* while preventing colonization by non-symbiotic bacteria. *V. fischeri* cells that enter the light organ must traverse a unique biogeography that includes ciliated ducts, an antechamber and a bottleneck. A single *V. fischeri* cell (or on occasion several cells) migrates into each of the three crypts where it becomes nonmotile and divides and grows until a cell density is reached that enables the induction of quorum sensing and light production (approximately 9–12 h). Colonization also initiates cellular changes in the host, including the induction of haemocyte migration into the host ciliated fields along with apoptosis and regression of the ciliated field 4 days after colonization. LBP, lipopolysaccharide binding protein; BPI, bactericidal permeability-increasing protein; NOS, nitric oxide synthase; NO, nitric oxide; PGRP2, peptidoglycan recognition protein 2. Scanning electron micrograph reprinted with permission from REF.[121], Oxford University Press.

We described what had been learned in those early years about a 'winnowing' process by which *V. fischeri* outcompetes other environmental bacteria through a series of interactions between the host and the co-evolved symbiont. In the years since, a collective effort from a growing number of laboratories has revealed not only many of the mechanisms that contribute to the initiation of the association (FIG. 2), but also many of those that mediate long-term maintenance through the ontogeny of the host. With the tremendous growth in the field, it would be lengthy to cover, even in broad strokes, what has been learned about this partnership from the vantage point of both host and symbiont. Thus, in this Review, we address the strides that have been made in recent years in the study of bobtail squid symbiosis from the host viewpoint; specifically, we explore how light-organ colonization influences early development and maturation of the host (FIG. 1). Our article is intended to complement the companion Review (see Visick, Stabb and Ruby[13]), which details advances over this same period in understanding the association from the perspective of the host's luminous partner *V. fischeri*.

Multi-omics
Multiple analysis approaches applied to a biological system, for example, genomics, transcriptomics, proteomics and metabolomics.

Holobiont
Also known as metaorganism. Refers to a host and all of its associated partners.

E. scolopes as a symbiosis model host

The bobtail squids and their associations with bacteria are naturally occurring binary (one host and one microbial species) symbioses that offer robust experimental systems (BOX 1) complementary to other more complex symbiosis models, such as *Hydra*, *Drosophila*, zebrafish and mice[5,14]. The squid's symbiotic associations exemplify a very common type of host–microorganism alliance in animals: one that is acquired anew each generation from the environment and characterized by the co-evolved microbial partners residing extracellularly along the apical surfaces of epithelial tissues[15]. The nocturnal squid host can easily be collected from the wild at night when it emerges to forage and can be raised through the complete life cycle[16]. As these animals are less active than many other cephalopod species owing to their benthic nature and limited diurnal activity, they are easily maintained in captivity with natural or artificial seawater and they adapt well to modified zebrafish or *Xenopus* aquaria. Captive females mate and lay serial clutches of eggs year-round, and a single egg clutch can yield hundreds of juveniles that hatch after a ~20-day embryonic period. More than 50,000 juveniles can be produced in the laboratory over a year by maintaining a population of about 12 female and 6 male animals.

The morphology of the light organ fosters emission of symbiont luminescence from the ventral surface of the squid host; it is hypothesized that the luminescence is used as camouflage to avoid predation[7]. As such, the authors have confirmed that the host is not physiologically compromised and requires no special care when maintained in the aposymbiotic state in the laboratory, that is, exposed to natural seawater without sufficient symbionts for colonization. Further, assessment and manipulation of the host symbiotic state can be carried out by measuring the light emitted by the light organ[6]. The relationship between luminescence output and symbiont number can be empirically defined for a given strain[6,17]; different strains of *V. fischeri* have different luminescent outputs in vivo[18]. Thus, host light emission not only defines the presence or absence of symbiosis by the wild type or light-producing mutants, but also enables an estimate of the relative degree of colonization in the light organ.

Several other features make the squid a compelling experimental subject. The ability to raise animals under aposymbiotic conditions throughout their life cycle has enabled researchers to determine crucial features of the association, such as the phases of symbiosis development and their associated morphological, physiological, biochemical and molecular signatures. The symbionts

Box 1 | **Key features and advantages of the *Euprymna scolopes–Vibrio fischeri* association**

The host *E. scolopes*
- easily obtained from the field
- is fecund year-round
- no parental care of eggs
- hatchlings live on yolk reserves and therefore do not need to be fed for colonization experiments
- small juveniles, entire animal viewable by confocal microscopy
- has capacity to be raised in the laboratory aposymbiotically (in natural seawater without symbionts)
- with effort, can be reared through multiple generations in the laboratory
- full genome sequence available
- omics of the light organ possible both within and between clutches; that is, at high and low genetic diversity

The symbiont *V. fischeri*
- free-living stage readily accessible
- culturable on minimal or defined media
- genetically tractable
- displays symbiosis-relevant strain variation
- dozens of full genome sequences available
- extracellular along apical surface of host epithelia
- physiologically well characterized; for example, quorum sensing
- related to important human pathogenic species

The *E. scolopes–V. fischeri* partnership
- ecologically obligate (aposymbiotic hosts not observed in the wild)
- binary and/or experimentally tractable
- natural mode of infection easily reconstituted in the laboratory
- symbioses not nutritional; hosts remain healthy without symbionts in the laboratory
- large number of juvenile hosts for experiments; robust statistics
- short time frame of initiation and early development
- luminous, non-invasive measure of symbiotic state
- maturation coupled to immunity and partner physiology
- has a complex daily rhythm
- antibiotics, fluorochromes, inhibitors and activators easily administered
- amenable to the study of space biology and microgravity experiments[122,123]

interface with two types of polarized epithelium, specifically ciliated and microvillous[6,8,19,20], which are the two most common types of epithelial mucosal surfaces associated with host–microorganism interactions across the animal kingdom. Because the light-organ epithelial tissues are exposed to the environment via the mantle cavity, the responses of the system to pharmaceuticals (for example, concentration and time of the response to exposure) are similar to what is observed in mammalian cell culture. In addition, the light organ can be quickly cured of its symbionts using low-dose antibiotics for a period of hours to days, depending on life stage; such experimental treatment enables the identification of reversible and irreversible symbiont-induced changes in the host organ[9,19–21].

The squid–vibrio association is also amenable to many different observational and experimental methods and techniques (BOX 1; TABLE 1). For example, in the light-organ symbiosis, because the newly hatched juvenile is ~2 mm in total length, one can observe and analyse the colonization process as the symbionts are harvested from the ambient seawater during the first hours after the juvenile emerges from the egg[22]. Once *V. fischeri* cells have been preferentially recruited onto the ciliated light-organ surface, the symbionts migrate into the tissues and traverse several distinct biomechanical and biochemical environments across an ~150 µm microbiogeographical landscape[12,23,24]. They reach their final destination of the microvillous crypt spaces by ~9 h (FIG. 2), although the timing of this can vary with different strains of *V. fischeri*. The transparency of this entire pathway permits real-time observation of the spatiotemporal relationship of the symbionts to host tissues over the trajectory of the colonization process. Perhaps one of the most powerful attributes of the light-organ association is that it is a model with well-developed genetic tools in the bacterial partner (see Visick, Stabb and Ruby[13]). As such, unlike symbioses with bacterial consortia, the squid–vibrio model, similar to the legume–*Rhizobium* association, is amenable to genetic manipulation in 50% of the partners (see below).

The winnowing and early development

Recruitment of the symbiont. In the past 15 years, researchers studying the squid–vibrio symbiosis have learned that the winnowing, from the species-rich bacterioplankton to the exclusively colonizing *V. fischeri* cells, is a complex biomechanical and biochemical process. Long cilia on the outer surfaces of the juvenile light-organ 'appendages' beat metachronally, a behaviour that focuses bacterium-sized particles and host-shed mucus to an area above the three pores on each side of the organ[25] (FIG. 2). Along the inner surface and radiating out from the base of the appendages are short, randomly beating cilia, the motion of which may mix the rich array of host immunity factors, principally antimicrobials, that are present in the chitin-rich mucus, as well as factors that are exported from the aggregating *V. fischeri* cells[25–33].

Under laboratory conditions similar to the natural environment, *V. fischeri* cells are recruited and these cells attach to the host cilia[34] as aggregates, where they signal a change in host gene expression across the entire organ[35]; the number of aggregating *V. fischeri* cells varies with strain[36]. In the host, genes encoding antimicrobials are upregulated, as are those encoding a chitinase[35] (FIG. 2). The data suggest that this activity has two priming functions in the mucus: to create a biochemical environment that selects for *V. fischeri* and prepares the bacterial cells for the increased antimicrobial environment of host tissues; and to break down the polymeric squid-generated chitin into chitobiose, which is the chemoattractant that draws would-be symbionts into host tissues[37]. Much study has gone into the characterization of a *V. fischeri*-secreted exopolysaccharide and regulation of its production, which is essential for efficient colonization, as is the ability to undergo normal chemotaxis (see Visick, Stabb and Ruby[13]).

The journey into host tissues and settling into a long-term symbiosis. After pausing in the aggregates, *V. fischeri* cells are then released from the superficial ciliated

epithelium and move into one of six pores, through a duct and into a broadened antechamber, the medial edge of which has a narrow bottleneck that leads to one of the six independent crypts[23]. On occasion, more than one *V. fischeri* recruit can enter each crypt space. However, on average and irrespective of the inoculum size, a single *V. fischeri* cell, which has traversed the narrow bottleneck, enters each crypt space and grows clonally[38,39]. Through quorum signalling, luminescence is induced about 12 h after inoculation of the seawater[40]. Analysis of the bottleneck region by confocal microscopy revealed that this is a highly dynamic region of the light organ[24]. It undergoes substantial constriction after the *V. fischeri* inoculum enters the crypts, which spatially restrict the symbiont populations over the day; during the daily venting of ~90% of the symbionts at dawn, the bottleneck briefly reopens[24].

Shortly after crypt colonization, the symbionts signal the cell-death-mediated decommissioning of the superficial ciliated surface that has potentiated symbiont colonization[21,41,42]. This process is induced by *V. fischeri* microorganism-associated molecular patterns (MAMPs) that are derivatives of the cell envelope (BOX 2), as well as by light production[41,42]. As the ciliated cells die, the activity of a matrix metalloproteinase and a cathepsin drives disruption of their basement membrane and their eventual detachment from neighbouring cells[29,43]. The symbionts affect this morphogenesis remotely, that is, from inside the crypt spaces[44]. Haemocyte infiltration and changes in host gene expression are essential for this

Table 1 | **Advances in techniques used to study the symbiotic associations of *Euprymna scolopes***

Technique	Tissues or cells analysed	Applications	References
Molecular			
Whole genome	Genomes of host and 62 *Vibrio fischeri* strains, including ES114 and MJ11; 13 D and S strains from *V. fischeri*; 16 ANG symbiont strains of *E. scolopes*	Discovery of novel genes important for symbiosis and host range; phylogenetic comparison of ANG strains and biosynthetic potential; identification of symbiosis-related host genes	18,38,58,105–109
Microarrays	*V. fischeri* and light organ	Comparisons of global gene expression in host and symbiont (13,982 ESTs)	64,110,111
RNA sequencing	Light organ and *V. fischeri*	Identification of host and symbiont gene expression during the first hours to days of the association	35,59,112
NanoString (nCounter Analysis)	*E. scolopes* and *V. fischeri*	Resolution of multiple genes with as few as 10 juvenile animals	59
Shotgun proteomics	Light-organ exudate and haemocytes	Identification of host and symbiont proteins in exudate and first description of innate immune-related proteins from haemocytes	86,113
Quantitative proteomics (iTRAQ and spectral counting)	Haemocytes (symbiotic and cured)	Identified quantitative differences in protein abundance in haemocytes	87
16S amplicon sequencing	ANG bacteria	Characterization of the bacterial community in ANG and associated eggs	114
Metagenomics	ANG	16S rRNA gene diversity of the consortium	115
INSeq	*V. fischeri*	Discovery of colonization determinants	116
Gene editing	*Euprymna* spp.	Knockout of host genes	[a]
Cellular			
Confocal microscopy	*E. scolopes* and *V. fischeri*	Live cell imaging of colonization; ultrastructure; cytochemistry; immunofluorescence	19,22,23,28,41,84
Transmission electron microscopy	*E. scolopes* and *V. fischeri*	Characterization of ultrastructure	6,17,115
HCR FISH	Light organ and *V. fischeri*	Cellular localization of gene expression in host and symbiont in time and space	47
In situ hybridization	Developing *E. scolopes* embryos	Allowed visualization of HOX gene expression in developing embryos	96
Imaging mass spectrometry	*V. fischeri* and host light organ	Imaging of small molecules in light organ	117,118
Physiological			
Molecular networking	Bacteria residing in the ANG and eggs	Chemical identification and modelling of secondary metabolites	119
Model-enabled gene search	*V. fischeri*	Identification of metabolic function pathways	120
Metabolomics	*E. scolopes*	Identification of host metabolites	78
NanoSIMS	*E. scolopes* and *V. fischeri*	Transfer and localization of metabolites	49

ANG, accessory nidamental gland; EST, expressed sequence tag; HCR FISH, hybridization chain reaction fluorescence in situ hybridization; INSeq, insertion sequencing; iTRAQ, isobaric tags for relative and absolute quantification; NanoSIMS, nano secondary ion mass spectrometry. [a]C. Albertin and J. Rosenthal, personal communication.

Box 2 | **Signalling and microorganism-associated molecular patterns**

Host–microorganism associations are often mediated through the recognition of microorganism-associated molecular patterns (MAMPs) by host pattern recognition receptors (PRRs). Many such molecular interactions occur in the squid–vibrio symbiosis. For example, peptidoglycan (PGN) and lipopolysaccharide (LPS) are required for initiating host mucus secretion and apoptosis, respectively, in the nascent light organ[19,41]. A key study demonstrated that a derivative of peptidoglycan (tracheal cytotoxin (TCT)) along with LPS trigger morphogenesis of the juvenile light organ[42]. Even though the structure of TCT is identical in the non-pathogenic symbiont *Vibrio fischeri* and the human pathogens *Neisseria gonorrhoeae* and *Bordetella pertussis*, it does not lead to virulence in the squid and is instead a signalling MAMP that is part of normal developmental pathways. Other findings showed that modification of the lipid A portion of LPS in *V. fischeri* is important for signalling to the host[27,124]. *V. fischeri* also releases LPS and outer membrane vesicles (OMVs) from flagella[50,51]. These OMVs can trigger development of the juvenile light organ independently of TCT[44]. For the host, studies have revealed several PRRs along with a conserved nuclear factor-κB (NF-κB) signalling pathway[86,110,125]. These PRRs include five peptidoglycan recognition proteins (PGRPs), several Toll-like receptors (TLRs), LPS-binding proteins (LBPs), two galectins and four bactericidal permeability-increasing proteins (BPIs)[26,28,80,81,86,87,110,125]. Some of these PRRs are expressed in specific tissues and cell types and change localization after colonization. For example, PGRP1 is present in the nuclei of host epithelial cells, but is lost in those cells later in development[80]. *V. fischeri* mutants that are defective in TCT release do not induce loss of PGRP1, suggesting that this MAMP can directly influence localization of this host PRR. PGRP2 is secreted into the light-organ crypt spaces and in mucus shed from the ciliated epithelium during initiation of the association[28]. Many of these PGRPs also have amidase activity[28,86], thus potentially enabling these PRRs to regulate MAMPs.

process[44–46], which suggests that these blood-borne cells may be the messengers. Finally, the presence of the symbionts in the crypts shuts down mucus shedding from the ciliated surface[19].

The presence of the symbionts affects the tissues along the pathway of colonization as well as those of the crypts. Both the duct and bottleneck constrict, and changes in gene expression occur along this migration path[24,47]. The epithelial cells lining the crypts, which interact directly with the symbionts, swell and increase the density of microvilli along their apical surfaces, a response that is also controlled by the MAMP lipopolysaccharide (LPS)[20,48]. Within the crypts, the symbiont population is exposed to many of the same antimicrobials as those that mediate initial colonization[26–33,35], which might shape or control the *V. fischeri* population.

Studies of the mechanisms of communication between host and symbiont have suggested both interactions at the symbiotic partner cell surfaces and uptake of symbiont products into host cells. A recent study that used quantitative ion microprobe imaging via Nano secondary ion mass spectrometry (NanoSIMS) showed that biomolecules from *V. fischeri* are taken up directly by the light-organ crypt epithelium and become enriched in the euchromatin and nucleoli of these cells[49]. The delivery mechanism of symbiont products is either by diffusion of molecules from the symbiont to the host cells or by host-cell uptake of outer membrane vesicles (OMVs) produced and released by the bacteria[44,50,51]. The ability of host cells to readily take up molecules generated by *V. fischeri* could be one mechanism by which symbiont signalling leads to some of the downstream cellular changes observed in the host. For example, a recent study demonstrated the abundant trafficking of a small RNA of *V. fischeri*, SsrA, into host cells[52]. This symbiont product profoundly affects the host's ability to form a normal symbiosis. Continuing studies of the role of OMV cargo promise to provide a clear picture of the transport of bacterial biomolecules into host cells. Finally, within 12 h after colonization, the resident symbionts also induce the onset of a profound daily rhythm in the host[53] (FIG. 3; see below).

In the absence of the symbiont. Most of the above-mentioned host immune and developmental phenotypes are defective in animals colonized by a non-bioluminescent (dark) mutant of *V. fischeri*, reflecting the fact that light production is the principal commodity that the host derives from the symbiont[54]. Morphological and molecular studies of the light organ have demonstrated that it shows remarkable convergence with the eye, including luminescence perception with the same gene products (for example, opsin, retinochrome and visual G proteins) that control environmental light perception in the retina[55–58] (see also description of findings from sequencing of the host genome below). Further, a recent study[59] that described the influence of light-organ colonization on the gene expression of remote tissues revealed that symbiosis induces changes in the transcriptome of the eye; studies with symbionts defective in light production showed that all changes in gene expression in the eye are dependent on the production of symbiont luminescence in the light organ. This correlation perhaps reflects the crucial coordination between the eye and light organ that mediates effective counterillumination, the antipredator behaviour of the host[59].

Finally, it should be noted that most of the work on the early stages of light-organ symbiosis used a single strain, *V. fischeri* ES114, as the colonizing symbiont. Recent studies have shown that symbiont strain variation can affect the processes of initiation[24,36,60]; therefore, like many other facets of the system, studying strain variation offers new opportunities (see Visick, Stabb and Ruby[13]).

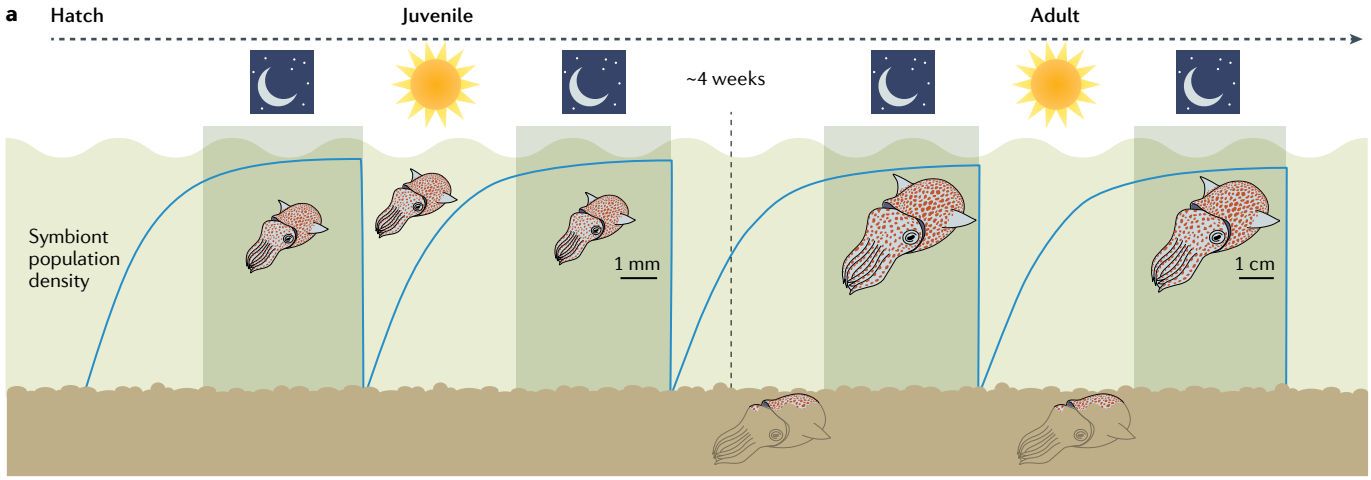
Symbiosis maintenance

The dynamic daily rhythms. Over the past decade, it has become clear that microorganisms have a profound influence on animal daily rhythms, affecting everything from metabolism to immune response[61,62]. The squid–vibrio system has been one of the principal experimental models for exploring this phenomenon (for example, see REFS[63,64]). Much of the work on the squid–vibrio system over the past three decades focused on the characterization of the early events of symbiosis, and the long-term maintenance of the association remained poorly understood. However, during the past 10 years, research on the system has demonstrated that one principal component of maintenance is the development of a profound daily rhythm of the association that begins with the onset of the symbiosis[40] and continues throughout the life of the host[64] (FIG. 3). This daily rhythm has two components, a diel feature (requiring a light cue each day for host response) and a circadian portion (a recurring endogenous cycling). In the former, the animal expels much of the crypt contents each day in response to the dawn light cue[65,66], including 70–95% of the symbiont population.

As mentioned above, this daily expulsion is concurrent with a widening of the bottleneck that helps to facilitate movement of the symbionts out of the crypts and into the antechamber and ducts in preparation for venting into the environment; the bottleneck then constricts again within 2 h after venting[24], confining the renewed cohort of symbionts that repopulate the crypts. In addition to the expelled symbionts, the vented exudate contains host cell debris and haemocytes that were present in the matrix that surrounds the bacterial cells within the crypts[66]. The later circadian component is reflected in cycling of behaviours and associated gene expression that occurs in anticipation of the light and dark cues[64]. This daily venting has at least three purposes: to seed the environment with viable *V. fischeri* that are symbiosis-competent and can colonize the next generation of bobtail squid[67,68]; to provide a way to renew the otherwise non-growing crypt population[64]; and perhaps to provide a mechanism to sanction or remove underperforming or cheating symbionts, such as dark mutants that do not produce light[69,70]. Early observations of these phenomena prompted in-depth studies that have characterized aspects of the molecular, cellular and physiological events occurring over the day–night cycle of the symbiosis.

Setting the early rhythms. In the juvenile host, the daily rhythm is simpler than that of the mature adult animals. These two life stages share the dawn venting, which in both is accompanied by an effacement of the microvilli

a



b

	Juvenile crypts		Adult crypts	
	Day	Night	Day	Night
Host	• Clock genes ↓ • Microvillar density ↓ • Antimicrobials (PGRP2, alkaline phosphatase, and galaxin) ↑	• Clock genes ↑ • Microvillar density ↑ • Antimicrobials (PGRP2, alkaline phosphatase, and galaxin) ↓	• Crypt pH neutral, haemocyanin-bound oxygen ↑ • Macrophage migration inhibitory factor ↑ • Haemocyte number low in crypts • Clock genes ↓ • Cytoskeleton genes ↓ • Oxidative stress genes ↑ • Osmotic control genes ↑	• Crypt pH acidic, haemocyanin-bound oxygen ↓ • Macrophage migration inhibitory factor ↓ • Haemocyte trafficking into crypts • Clock genes ↑ • Cytoskeleton genes ↑ • Oxidative stress genes ↓ • Osmotic control genes ↓
Symbiont	• Regrowth complete (6–8 h post dawn) • Luminescence ↓	• Luminescence ↑	• Anaerobic respiration of glycerol phosphate • Regrowth complete (6 h post dawn) • Luminescence ↓	• Chitin fermentation • Luminescence ↑

Fig. 3 | **The diel rhythm of the host–symbiont association highlights major differences between juvenile and adult stages. a** | Within the first 4 weeks after hatching, the host transitions to a fully nocturnal active period with a functional symbiosis that allows for the camouflage behaviour (counterillumination) and a diurnal quiescent period when the host buries itself in the sand. Symbiont density is also regulated on a diel cycle where 95% of the symbionts are expelled each morning at dawn followed by a period of regrowth so that the host has a full complement of *Vibrio fischeri* to engage in counterillumination via bioluminescence at night. **b** | Host and symbiont gene expression are regulated as part of these crucial diel and circadian rhythms that drive the cell biology and biochemistry of the light organ to facilitate light production when the host is active at night. The symbiont diel population and corresponding luminescence rhythms are established early in the association, as are some crucial host pathways, including changes in microvillar density and in genes that help to regulate circadian rhythms and immunity. By 4 weeks, major cellular and physiological shifts occur in the light organ over the day–night cycle as indicated by changes in genes and/or proteins involved with osmolarity, oxidative stress, actin rearrangement, circadian rhythms and haemocyte trafficking. This haemocyte migration into the crypts at night helps to deliver chitin to the symbionts — a process that involves the symbiont undergoing a major metabolic shift, using the anaerobic respiration of glycerol phosphate during the day and fermentation of host-derived chitin at night that helps facilitate effective luminescence by acidifying the crypts and releasing oxygen from bound haemocyanin. PGRP2, peptidoglycan recognition protein 2.

from the apical surfaces of the crypt epithelial cells. Over the following hours, the apical surfaces of these cells repolarize, and the bacterial cells that remain behind after venting proliferate to fill the crypt spaces. Earlier studies of the crypt epithelia in juveniles showed that symbiosis induces an increase in microvillar density[20]. Microscopy analyses of the juvenile light organ over the course of this daily rhythm demonstrated that crypt epithelia of both aposymbiotic and symbiotic juveniles efface (effacement occurs in response to the light cue at dawn and not colonization)[48]. Whereas the microvilli of aposymbiotic animals repolarize to the same density, the presence of the symbionts induces an increase in microvillar density with each day. By day 4, the bacterial cells are surrounded by host microvilli[20], increasing the surface area between host and symbiont cells. An experimental study of this phenomenon demonstrated that the symbiont MAMP LPS is responsible for this increase in microvilli[48]. Also, over the day–night cycle in both juvenile and adult animals, the expression of the host *cry* gene cycles[53]. The *cry* genes encode a blue-light receptor protein that is a component of the molecular clock of animal circadian rhythms, and typically their transcription is highest at peak ambient light regimes. The light-organ *cry* isoform, *escry1*, cycles with the same timing as symbiont luminescence, peaking in the evening hours. This finding was consistent with early work on the system that showed that per-cell luminescence of symbionts in the organ cycles out of phase with environmental light; that is, it is brightest at night when the animal requires symbiont luminescence for its behaviours[71]. Experiments with juvenile animals demonstrated that the presence of light-producing symbionts is essential for *escry1* cycling[53].

In juvenile and/or adult light organs, a set of antimicrobial genes are also regulated on the day–night cycle, for example, those encoding peptidoglycan recognition protein 2 (PGRP2)[28], alkaline phosphatase (AP)[27], galaxin[31], cathepsin L[29], complement protein C3[32,64] and the squid's blood pigment haemocyanin, which has a C-terminal peptide with antimicrobial activity[30]. Two of these proteins, PGRP2 and AP, which detoxify the peptidoglycan monomer (also called tracheal cytotoxin (TCT)) and LPS of the symbionts, respectively, are not detectable in the early hours of the symbiosis. These bacterial 'toxins' (BOX 2) are the agents that mediate the induction of light-organ morphogenesis, which is irreversibly triggered at 12 h, so their detoxification before that point would compromise the normal symbiosis-induced developmental programme. Thereafter, the genes that encode these proteins cycle over the day, and the proteins can always be detected in the light-organ crypt spaces. However, they are not always active; in the adult, but not in juvenile animals, the crypt spaces change pH over the day–night cycle[63]. The pH optima of both PGRP2 and AP are at around 8 and, whereas the adult host cells would be protected by these proteins during the day when the pH is near neutral, the crypt acidifies in the late night. This acidification would diminish the activity of PGRP2 and AP in the hours preceding the dawn light cue for venting, rendering the host cells susceptible to perturbation by the intact MAMPs.

Maturation of the rhythms. Daily rhythms associated with the symbiosis are more pronounced and complex in the adult, in which they were first discovered in a study of the daily patterns of gene expression[64]. This analysis showed that both the host and the symbiont transcriptomes fluctuate, an oscillation reflected in a cellular restructuring of the host crypt epithelia, which is much more extensive in the adults, and in a change in symbiont metabolism over the day–night cycle. These studies characterized the 'central core' of the light organ, which comprises both the symbiont cells and the epithelial tissues that support them, at four time points over the day: 04:00 h, when the light-organ crypts are full of symbionts (just before the dawn expulsion); 10:00 h, when the animal is quiescent and the remaining symbiont cells grow to repopulate the crypts; 16:00 h, in the hours before the host emerges from the sand to use symbiont bioluminescence during foraging; and 22:00 h, when animal activity and symbiont luminescence are at their peak levels. These data revealed that gene expression changes in the host are most pronounced before and after dawn, around the time of the above-mentioned restructuring and repolarization that is associated with expulsion of the symbionts. Among the host genes regulated are all those recognized as encoding cytoskeletal proteins; at 04:00 h, expression of these genes is higher than at 22:00 h and, by 10:00 h, expression is lower than at 04:00 h. Concomitant ultrastructural analyses revealed that at 04:00 h, animals have highly disrupted epithelial cells; that is, the microvilli are effaced from the apical surfaces, the tight junctions are absent or abbreviated, and portions of the cells have blebbed into the crypt spaces; by 10:00 h, the cells repolarize. Given that effacement is triggered by a light cue in juvenile animals[48], the increase in expression of cytoskeletal genes in adults at 04:00 h may be anticipatory to the venting event at dawn.

There are several genes regulated similarly to the host cytoskeletal genes that encode antimicrobial proteins that may be protecting the animal from invasion of other tissues by *V. fischeri* when the integrity of the crypt epithelia is compromised. Large changes in symbiont gene expression follow those of the host. Bacterial activity over the day–night cycle has consequences for the host. Notably, as mentioned, the crypts acidify in the hours before venting. Haemocytes from the host circulatory system migrate into the light organ where a certain number of these cells lyse, releasing chitin stores that the symbionts metabolize by fermentation[63]. A recent study demonstrated that movement of the haemocytes into the crypts is regulated by a cytokine, macrophage migration inhibitory factor[72]. This immune protein is abundant in the symbiotic tissues during the day, perhaps to deter migration of the haemocytes into those regions, and is in low abundance at night, enabling haemocytes to traffic into the crypts to release their chitin stores. Thus, this cytokine is controlling the provision of nutrients to the bacterial symbionts. Further, the major oxygen-binding respiratory protein of the squid, haemocyanin, traffics into the crypt spaces where it would offload its oxygen more readily under the acidic conditions, thereby providing the symbionts with the oxygen that is crucial for the luminescence reaction[30]. After the effacement of

the host cells, the symbionts increase expression of the genes associated with anaerobic respiration of glycerol phosphate[64], a pH-neutral process, and the crypt loses its acidic nature. The data support the model that the bacteria are using membranes of the host animal for their regrowth in the crypts, as the membrane signature of the symbionts in the crypts includes fatty acids produced only by the host animal[64]. Once the membranes are depleted by symbiont growth and the host cells repolarize, the expression of genes associated with the use of membranes for nutrition decreases. Then, the symbionts express the genes associated with chitin fermentation, an acidifying metabolism, which suggests that the host is supplying the symbionts with this polymer as the subsequent nutrient source.

Beyond the daily rhythm

Advances in husbandry. Characterization of long-term development has been facilitated by major strides in husbandry efforts to raise *E. scolopes* reliably to sexual maturity[16,73,74]. These advances have enabled researchers to move beyond either the first 4–5 days when the light-organ association is experimentally initiated, or the use of field-caught adults, and ask: how symbiosis influences long-term development of the host; and what are the mechanisms by which a stable association is maintained. Although the presence or absence of accessory tissues (that is, lens, reflector and filters) does not seem to depend on light-organ colonization, several physiological, biochemical and molecular changes occur concomitantly with a transition of the animal from the juvenile to the mature state[16,59,75]. One of the most obvious transitions is in host behaviour; after 4 weeks, the animals transition between two distinct patterns: an active nocturnal period and a quiescent diurnal period[16,73]. The ability to raise the animals has also enabled the study of the window of colonization.

Although in the environment colonization is likely to occur within hours after hatching, in the laboratory setting, the host can be receptive to colonization by *V. fischeri* for at least 4 weeks after hatching. Earlier studies using symbiotic juveniles found that if colonized hosts are treated with antibiotics within 5 days to remove the symbionts, then the light organ can be recolonized[19,21]. However, if the light organ is colonized by the symbiont for at least 5 days followed by curing, the host enters a refractory state and subsequent colonization does not normally occur[16]. This refractory period seems to be refined to restrict further colonization by other *V. fischeri* cells. By contrast, animals that are colonized for up to 10 days by a dark *V. fischeri* mutant that is defective in light production (Δ*lux*) can be recolonized, which suggests that the host has the ability to restrict strains that are underproducing the currency of the association (that is, light). Morphologically, the superficial ciliated epithelium of aposymbiotic animals (FIG. 1) persists for up to 1 month but undergoes different degrees of regression, perhaps owing to constant exposure to low levels of exogenous MAMPs[16,75] produced by the non-symbiotic bacterioplankton. Despite a loss of the ciliated epithelium, aposymbiotic animals can generally be colonized within the first month with high densities (>5,000 cells per ml) of *V. fischeri* in the environment. Taken together, work with juvenile and adult hosts suggests that, although the superficial ciliated epithelium facilitates colonization, it is not essential for the colonization process.

Other morphological developmental events include a change in pore number on the surface of the light organ[75]. Aposymbiotic animals maintain three independent pores on the surface, whereas the pores of symbiotic animals coalesce into a single pore by 4 weeks. Animals colonized with a *V. fischeri* Δ*lux* mutant show intermediate morphology[75]. Analysis from this same study concluded that, compared with symbiotic animals, the average length and density of cilia associated with the superficial epithelium is greater in animals raised aposymbiotically. A comparison of 4-week-old aposymbiotic or wild-type colonized animals, with animals colonized by the Δ*lux* strain, also revealed that the epithelial cells of the light-organ crypts change from the columnar morphology characteristic of aposymbiotic or Δ*lux*-colonized animals, to cuboidal in wild-type colonized animals[75]. These observations are consistent with work in juvenile hosts that showed an increase in crypt epithelial cell volume and microvillar density within the first 24 h after colonization[20]. Therefore, these early symbiont-induced phenotypes persist from the juvenile into the adult stages.

A comparison of light-organ gene expression among aposymbiotic, wild-type colonized and Δ*lux*-colonized animals after 4 weeks supports the morphological changes noted above[75]. For example, the expression of genes associated with osmotic regulation and fluid uptake is increased in symbiotic animals, consistent with the observation of an increase in crypt-cell swelling after colonization. Genes associated with signalling are over-represented in symbiotic animals, whereas those associated with a response to oxidative stress remain unchanged or are poorly expressed. Common immune pathways also seemed unchanged between aposymbiotic and symbiotic animals after 4 weeks. Together, these data suggest that the host undergoes a transition that facilitates a persistent accommodation of the symbiont, albeit one that is dynamic on a daily basis.

Light-organ colonization influences systemic host gene expression. The long-term effects of light-organ colonization also influence gene expression in remote tissues, specifically the eyes and gills[59]. The influence of microbiomes on remote tissues is currently a highly active area of research; for example, the gut–brain axis in mammals[76]. In the squid–vibrio association, the eye was chosen as a study subject because it shows a remarkable convergence with the light organ, from morphology to biochemistry[55–57,77], presumably because both organ types function to modulate light. By contrast, the gill tissue was selected for comparison because it has an immune function in squids and their close relatives[59], and responds to bacterial infection. Experiments with the Δ*lux* mutant of *V. fischeri* showed that most of the regulated light-organ genes respond to symbiont light production rather than the presence of the symbiont itself. Both eye and gill tissue also exhibit changes in gene expression as a result of light-organ colonization, but almost no overlap was

Metabolome
Collective metabolites associated with a given organism or organisms.

observed in responsive genes among the three organs. Amazingly, the eye lost its entire response to symbiosis when the host was colonized by a dark mutant of *V. fischeri*, demonstrating that the eye's response is exclusively due to the symbiont's bioluminescence. By contrast, the gene expression patterns in the gill in response to colonization was the same in wild-type or Δ*lux V. fischeri*, demonstrating that the gill responds to the presence of bacteria in the light organ instead of to the symbiont's light production. Change in many of the genes that are regulated on a daily rhythm persists as the host matures from juvenile to adult[59]. A recent study that analysed the metabolome of adult haemolymph showed that light-organ colonization influenced approximately 25% of the metabolites present in the blood of adult males and that the levels of these molecules fluctuated on a diel cycle[78]. Future studies will help to uncover the systemic effects of bacterial colonization on an animal host.

The effect of symbiosis on innate immunity

Symbiosis with microorganisms can influence several developmental pathways in eukaryotes, including that of the immune system. The ability to either maintain animals as aposymbiotic or use antibiotics to cure the host has revealed several ways that *V. fischeri* influences both cellular and acellular components of the host's immune system during development of the association. In particular, symbiosis influences the production of reactive oxygen and nitrogen species[33,79], components of the complement pathway[32], antimicrobial proteins such as haemocyanin[30], peptidoglycan recognition proteins[28,80], and proteases and LPS-binding proteins[81].

One other advantage of *E. scolopes* as a model host is that it relies solely on an innate immune system and that it has only one type of circulating immune cell, a macrophage-like haemocyte. Therefore, understanding the influence of symbiosis on cellular immunity is not confounded by responses of an adaptive immune system or of multiple blood cell types. Like other haemocytes of invertebrates, these cells have macrophage-like properties capable of binding and phagocytosing bacteria and other microorganisms[82–84]. Because bobtail squid have a closed circulatory system and the light organ is highly vascularized, haemocytes can traverse through the surrounding superficial epithelia and enter the crypt spaces in both juveniles and adults[45,66].

Several studies have focused on the ability of haemocytes to differentiate between *V. fischeri* and other non-symbiotic species with regard to binding and phagocytosis. Immediately after hatching, juvenile haemocytes display differential phagocytosis between *V. fischeri* and non-symbiotic *Vibrio harveyi*[85]. Despite binding to both bacterial species equally, only *V. harveyi* is engulfed. The ability of haemocytes to bind bacteria increases over the first 4 days after hatching independently of the colonization state of the light organ[85]. However, as the host ages, the haemocytes seem to undergo maturation, leading to differential binding and phagocytosis depending on the light-organ colonization state. Haemocytes from symbiotic adult animals bind fewer cells of *V. fischeri* than non-symbiotic *V. harveyi* and *Photobacterium leiognathi*[84]. Although bound bacteria are also phagocytosed, haemocytes from aposymbiotically raised adult animals bind more, but phagocytose substantially fewer *V. fischeri* cells than those from symbiotic animals; thus, the haemocyte response of hosts raised without the symbiont more resembles that of juvenile squid[85]. However, haemocytes from symbiotically raised and wild-caught animals bind fewer *V. fischeri*, yet phagocytose more *V. fischeri* overall[85]. Further, curing the adult light organ with antibiotics alters the binding of haemocytes to *V. fischeri*, resulting in a significant increase in binding to a level similar to that of *V. harveyi*[84]. Taken together, these juvenile and adult studies suggest that initial haemocyte recognition of the symbiont occurs principally at the level of phagocytosis, whereas haemocyte-binding dynamics are more important later in development.

The mechanisms behind this immune maturation are unknown, but transcriptomic and proteomic studies of haemocytes from symbiotic and cured hosts have revealed potential targets that may be involved in the recognition of bacteria, including pattern recognition receptors (PRRs) such as peptidoglycan recognition protein 5 (PGRP5) and galectins[86–88]. In cephalopods, haemocytes develop in the white body, and gene expression analysis in a related sepiolid squid species supports the notion that this organ is an active site for haematopoiesis[89]. Future analyses will focus on whether colonization of the light organ by *V. fischeri* influences haematopoiesis remotely, leading to downstream effects on haemocyte binding and phagocytosis. *V. fischeri* is likely to also exhibit mechanisms to actively avoid haemocyte recognition, as a strain with a mutation in an outer membrane porin (OmpU)-encoding gene binds statistically significantly more to haemocytes, and in a manner similar to the non-symbiotic species *V. harveyi* and *P. leiognathi*[84].

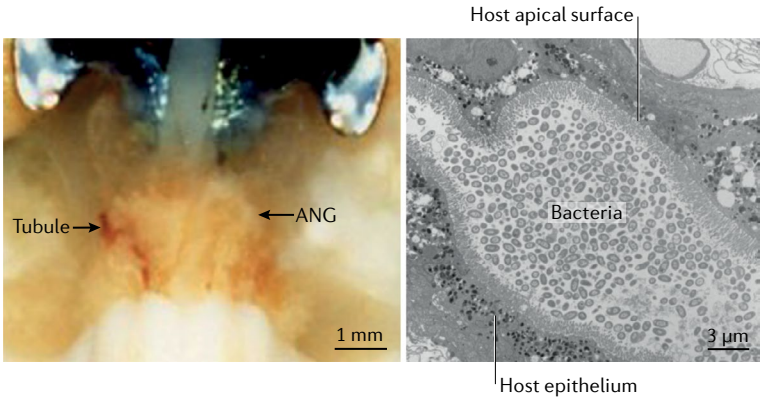
Conclusions and future directions

The squid–vibrio symbiosis is one of a growing number of model associations that have revealed general principles to help inform basic biology and biomedicine about the form and function of host–microorganism interactions (reviewed in REF.[15]). The use of models has been essential for the study of highly complex features of animals and plants, such as developmental biology and neurobiology, revealing patterns of evolutionary conservation and diversification. Every model association brings its unique strengths to microbiome research. As human symbioses involve complex consortia, studies of both simple and complex consortia, typically in invertebrates and vertebrates, respectively, are highly valuable to biomedicine. However, studying the role of all partners in a natural, co-evolved system is currently only possible with binary or simple consortial symbioses. The oldest and best understood of the binary relationships is the symbiosis between leguminous plants and rhizobia, which have provided foundational knowledge for the field[90].

From the outset of research on the squid–vibrio system, the highly conserved nature of interkingdom interactions was demonstrated with comparisons drawn between the squid–bacteria and legume–bacteria symbioses[91]. For decades the legume–rhizobia symbioses

provided the only genetic model for studying symbiotic associations. Similar to the squid–vibrio system, genetics was only available in the bacterial partners for the early studies of plant associations. However, in the past 20 years, genetics have been developed in the host plant, rendering these symbioses the only available models with high-quality genetics in all partners[92]. Development of genetics is on the horizon for the squid host, which promises to provide an analogous system and strong complement to the legume–rhizobia associations. Nonetheless, with genetics largely restricted to the microbial partner, the squid–vibrio symbiosis has provided valuable insights into the basic nature of animal symbioses, helping to broaden our conceptual framework of the field. Particularly valuable for biomedicine, the squid–vibrio system offers the opportunity to explore the interaction of extracellular Gram-negative bacteria with epithelial surfaces, the principal type of host–microorganism relationship in humans[93]. For example, work from the squid–vibrio association

Box 3 | **The symbiosis of the accessory nidamental gland**



Although for more than 30 years research on symbiosis in *Euprymna scolopes* has focused principally on the binary light-organ partnership, a second association involving a bacterial consortium and the accessory nidamental gland (ANG) of female animals is now being intensely investigated. The ANG is an organ common to several cephalopods (see the figure, left panel), and comprises epithelium-lined tubules that, in contrast to the light organ, harbour a low complexity consortium (see the figure, right panel). In *E. scolopes*, this consortium is dominated by culturable roseobacters (Alphaproteobacteria), as yet uncultivated Verrucomicrobia and some culturable Flavobacteriia and Gammaproteobacteria species[114,115,126]. Approximately 50 core operational taxonomic units (OTUs) belonging to these taxa have been described from wild-caught populations of *E. scolopes*[114], representing approximately 80% of the ANG community. Each tubule of the ANG is dominated by a specific bacterial taxon[115], but the nature of this segregation is not understood. It may suggest that specific tubule microenvironments promote the growth and selection of different types of symbiont, or there are founder effects (that is, the microorganism that colonizes the space first proliferates and remains the dominant competitive species in that space). Haemocytes also traffic into the ANG tubules, although whether they have a similar role as in the light organ has not been determined[115]. As with the light-organ symbiosis, the ANG symbionts are environmentally transmitted at each host generation[114,127]. The cephalopod ANG develops approximately 1–2 months after hatching and is poised to recruit bacteria from the environment[127]. Whether, as compared with the light-organ association, *E. scolopes* uses similar mechanisms to ensure specificity of ANG colonization remains to be determined.

During oviposition, bacteria from the ANG are transmitted directly into an egg jelly coat that surrounds the embryo. Full genome sequences have been acquired for several of the ANG symbionts[106–109], which affords the opportunity to analyse symbiont genomics, metagenomics and metatranscriptomics of both the ANG bacteria and the colonized eggs. The resulting data have provided insights into the functional features of the association centred on the symbionts providing defence against pathogens and biofouling microorganisms during embryogenesis. Bacteria isolated from the ANG and/or eggs of cephalopods exhibit antibacterial activity in vitro, and antimicrobial compounds have been described from these strains[106,107,128]. A landmark study in *E. scolopes* showed that eggs that are cured of their symbionts through antibiotic treatment are more susceptible to fungal infections[119]. Bacterial isolates and their organic extracts from the ANG and eggs inhibit these fungi, and secondary metabolites were identified that may have a role in egg defence[119]. Mechanisms to prevent biofouling and/or pathogenesis in externally laid eggs have been described in other aquatic systems[129–131], and the ANG association in *E. scolopes* provides an emerging model to study the influences of microbiota in defensive symbioses.

Finally, a study of the whole genome of *E. scolopes* provides insight into cephalopod evolution and how symbiosis drives the evolution of organs that house microbiota[58]. These analyses show that the light organ and ANG are likely to have evolved via different mechanisms. Analysis of the host genome and light-organ transcripts supports findings of previous studies that light-organ physiology and biochemistry are most similar to the eye[54]. These results, along with previous studies[54,55,57,100], suggest that subfunctionalization of genes expressed in the eye had an important role in the evolution of the light organ. Gene expression analysis of tissues from adult hosts showed that the ANG has a high number of *E. scolopes*-specific transcripts (>35%). Analysis of the genomic regions surrounding these 'orphan' genes showed a greater proportion of repetitive-element content compared with genes expressed in other tissues, which suggests a high evolutionary turnover in genomic regulatory regions associated with the ANG. Expression of unique or taxonomically restricted orphan genes is hypothesized to contribute to genetic novelty in other animal groups[132,133]. The *E. scolopes* genome and patterns of the ANG transcriptome support this hypothesis, and future genomic and transcriptomic data sets from additional ANG-containing cephalopods will enable broader comparative approaches that will inform a better understanding of the evolution of this organ. Left panel reprinted with permission from REF.[66], University of Chicago Press; right panel reprinted from REF.[134], Springer Nature Limited.

demonstrated the importance of MAMPs in signalling to animal hosts, a phenomenon that is crucially important in both mutualistic and pathogenic associations[42] (BOX 2). In this benchmark paper, the authors changed the lexicon from 'pathogen-associated molecular patterns' to 'microorganism-associated molecular patterns' (that is, from PAMPs to MAMPs), with the recognition that these molecules are general symbiont cues of host–bacteria communication, independent of the type of association. Similarly, the squid–vibrio system has also been at the forefront of studies of symbiont-driven daily rhythms, a phenomenon that recent studies have shown is also important in humans and other mammals[94]. As presented in the companion review[13], many discoveries of *V. fischeri* have also contributed to our general understanding of the biomedical ramifications of animal–bacteria associations. Beyond the field of symbiosis, *E. scolopes* and related sepiolid species are emerging model organisms for the study of other areas of biology, including cephalopod genomics[58,95], the evolution of developmental mechanisms[96–99] and behaviour[100–103].

Over the past 30 years, the squid–vibrio association has revealed mechanisms by which environmentally transmitted symbioses are established and maintained. The experimental tractability of the association and the ability to manipulate the partners has helped to elucidate the intricate molecular conversation between the partners. One exciting new area of research in *E. scolopes* is the study of a second symbiosis — a bacterial consortium found in the accessory nidamental gland (ANG) of female hosts (BOX 3). Although the mechanisms of colonization in the ANG association are not yet as well understood as those of the light organ, the presence of two experimentally tractable symbiotic systems in the same genome-defined host now enables the identification and comparison of common and divergent mechanisms of colonization and maintenance in both binary and consortial associations. New advancements in omics, microscopy and animal husbandry have also moved this field forwards, facilitating a greater understanding of the specific features of these associations (TABLE 1).

These advancements and other emerging techniques present exciting opportunities to expand the use of the Hawaiian bobtail squid as a model organism in several areas of biology and medicine. For the study of both the light organ and ANG associations, the recent completion of the host genome sequence offers exciting avenues for research and tool development in *E. scolopes*[58]. This key advance provides the opportunity to define how the blueprint of the animal host shapes and has been shaped by its symbiotic associations. For example, it will now be possible to study gene regulation in female and male squid in the presence and absence, respectively, of the ANG consortium. Also, CRISPR–Cas9 technology for genetic manipulation of cephalopods is currently being developed[104], including in *Euprymna* spp. (TABLE 1); such an advance will enable researchers to interrogate the function of candidate genes that have been identified by studying natural variation in the host. In addition, many emerging tools are available for the study of the light-organ symbiont, *V. fischeri* (see Visick, Stabb and Ruby[13]).

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