CHAPTER ELEVEN

Animal development in the microbial world: The power of experimental model systems

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Abstract

The development of powerful model systems has been a critical strategy for understanding the mechanisms underlying the progression of an animal through its ontogeny. Here we provide two examples that allow deep and mechanistic insight into the development of specific animal systems. Species of the cnidarian genus *Hydra* have provided excellent models for studying host-microbe interactions and how metaorganisms function in vivo. Studies of the Hawaiian bobtail squid *Euprymna scolopes* and its

Current Topics in Developmental Biology, Volume 141
ISSN 0070-2153
https://doi.org/10.1016/bs.ctdb.2020.10.002

luminous bacterial partner *Vibrio fischeri* have been used for over 30 years to understand the impact of a broad array of levels, from ecology to genomics, on the development and persistence of symbiosis. These examples provide an integrated perspective of how developmental processes work and evolve within the context of a microbial world, a new view that opens vast horizons for developmental biology research. The *Hydra* and the squid systems also lend an example of how profound insights can be discovered by taking advantage of the "experiments" that evolution had done in shaping conserved developmental processes.

1. Models systems as a strategy for understanding conserved features of complex processes like development

"For a large number of problems there will be some animal of choice on which it can be most conveniently studied … I am afraid that most of them are unknown … Zoologists need to find them and lay their hands on them" (Krogh, 1929). Even before this insight by Krogh and in the years to follow, much has been learned about development through the study of animal models, a small set of diverse metazoans that have particular advantages for laboratory research. Research with model systems (Fig. 1) has shown a remarkable degree of similarity in the developmental mechanisms of all animals (see A. McKenna et al., this volume). These findings demonstrate that, although the embryology of simpler animals might appear superficially very different from that of humans, knowledge gained from those models can often be applied directly to understanding human developmental mechanisms.

Fortuitously, many of the important model organisms for developmental biology have also been adopted for the study of host-microbe interactions, including *Hydra*, the fruit fly, worm, zebrafish and mouse (Fig. 1) (reviewed in Bosch, Guillemin, & McFall-Ngai, 2019). In addition to these systems, other experimental model associations have come from a decades-long history in the field of symbiosis (e.g., the parasitic nematode-*Xenorhabdus* association, the squid-vibrio association, the leech) or have developed anew (e.g., bean bug, honeybee, starlet anemone) (Fig. 1). These models are being used to explore the development of the many animals that exist and function only in cooperation with an abundance of microorganisms. While some animals may be just an accidental place for microbial settlement, others appear to have coevolved with microbes over the course of the earth's history. Information on microbial colonization of animal tissue and documentation

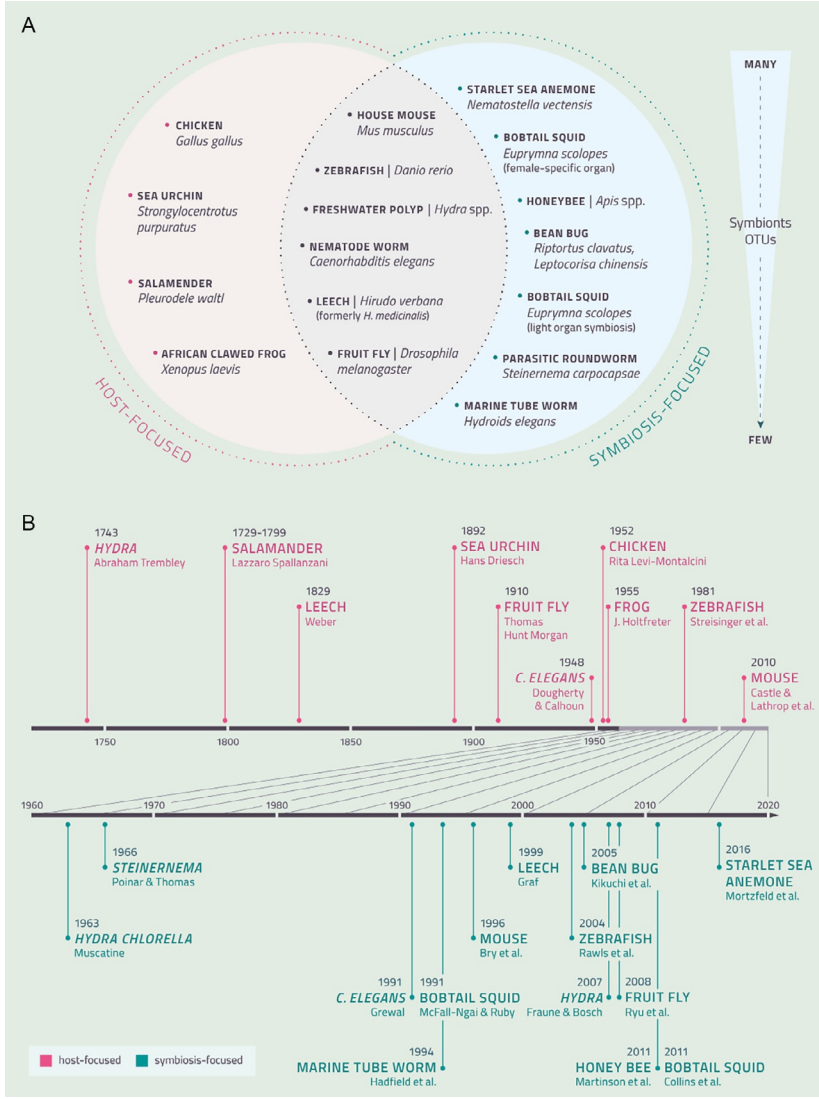

Fig. 1 Models of animal development. (A) Some traditional and new experimental model systems for the study of host-microbe interactions. Traditional systems (left circle) not widely used or widely used to study how symbiosis influences development; new models (right circle) with a major focus on the impact of symbiosis on development. (B) The timeline shows the date of publication of the corresponding model animal along with the discoverer (red lines: animal models for host-focused research; green lines: animal models for symbiosis focused research). *Traditional models of general development/model of microbe-induced development*: *Mus musculus*, mouse, Davisson & Linder (2004)/Bry, Falk, Midtvedt, et al. (1996); *Danio rerio*, zebrafish, Streisinger, Walker,

(Continued)

of physically associated taxa, including those that are coevolving with or codependent on the animal host, is still fragmentary because the field is young and only a few animal/plant/fungal species have been analyzed in depth. Microbes certainly are abundant and important to host fitness in corals, aphids, cows and in humans. Some other animal groups appear to harbor few or no resident microbes (table 1 in Hammer, Sanders, & Fierer, 2019). And in other animal taxa (including nematomorphs, entoprocts, gnathostomulids, gastrotrichs, brachiopods, phoronids, onychophora and others) nothing thus far is known at all about the microbiome.

Here we provide two examples of how interactions with microbes influence animal development. Our intention here is not to be exhaustive in the descriptions of the findings for each system, but rather to give the reader a flavor of how the study of models is providing insight into basic processes of host-microbiome associations and their impact on development. It should be emphasized that, as in traditional developmental biology, no single model system will be capable of addressing all questions in the field (Ruby, 2008). Rather the most powerful outcome will arise from the use of as many models of developmental processes that can be found in nature to reveal both conserved elements as well as those that create diversity in developmental of symbiotic systems.

2. *Hydra*: A model for host-microbe interactions—And a window into early animal life

The freshwater polyp *Hydra* (Fig. 2A and B) is an excellent model for studying host-microbe interactions and how metaorganisms function in vivo (Bosch, 2013, 2014; Klimovich & Bosch, 2018; Schröder

Fig. 1—Cont'd Dower, et al. (1981)/Rawls, Samuel, and Gordon (2004); *Caenorhabditis elegans*, nematode worm, Dougherty and Calhoun (1948)/Grewal (1991), Schulenburg and Félix (2017); *Hydra* spp., Trembley (1744)/Muscatine and Lenhoff (1963) (algal symbiosis), Fraune and Bosch (2007) (bacterial symbioses); *Hirudo medicinalis*, leech, Weber (1829)/Graf (1999); *Drosophila melanogaster*, fruit fly, Bellen, Tong, and Tsuda (2010)/Ryu, Kim, Lee, et al. (2008). *Symbiosis focused models*: *Nematostella vectensis*, starlet sea anemone, Mortzfeld, Urbanski, Reitzel, et al. (2016); *Euprymna scolopes*, bobtail squid (consortial symbiosis in females), Collins and Nyholm (2011); *Apis* spp., honey bee, Martinson, Danforth, Minckley, et al. (2011); *Steindernema* (formerly *Neoaplectana*) *carpocapsae*, nematode worm, Poinar & Thomas, 1966; *Riptortus clavatus* and *Leptocorisa chinensis*, bean bug, Kikuchi, Meng, and Fukatsu (2005); *E. scolopes*, bobtail squid (light-organ symbiosis), McFall-Ngai & Ruby (1991); *Hydroides elegans*, marine tube worm, Hadfield, Unabia, Smith, and Michael (1994).

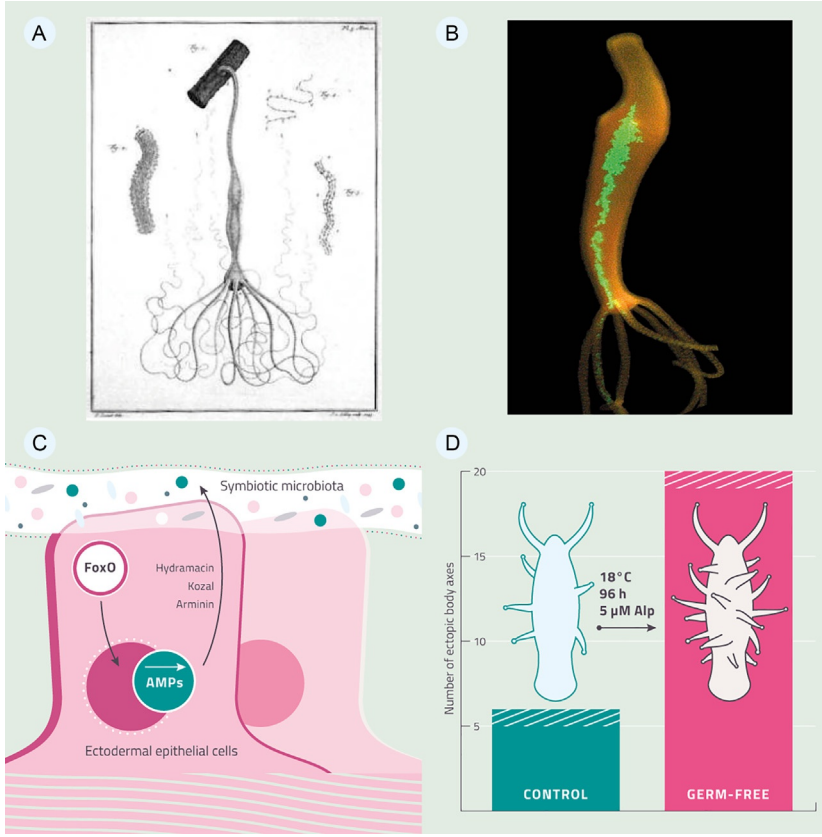

Fig. 2 The freshwater polyp *Hydra*. (A) A *Hydra* as depicted in Trembley (1744) book. (B) Transgenic *H. vulgaris* (AEP) polyp with a mosaic expression of EGFP in some of its endodermal epithelial stem cells. (C) Schematic representation showing the effect of transcription factor FoxO on expression of antimicrobial peptides (AMPs) and maintenance of the symbiotic microbiota. (D) WNT signaling-mediated developmental processes are dependent on microbial colonization.

& Bosch, 2016). In *Hydra*'s simple tube-like body structure, the single layer of ectodermal epithelial cells covered by a multilayered glycocalyx represents a physical barrier toward the environment, whereas a single layer of endodermal epithelial cells separates the body from the content of the gastric cavity. Both, ecto- and endodermal epithelial cells produce a rich repertoire of antimicrobial peptides that regulate the microbiome. In addition, ectodermal epithelial cells synthesize an extracellular cuticle ("glycocalyx") with a complex layered structure consisting of several proteins and glycosaminoglycans. The outer layer of the glycocalyx has mucus-like properties and

provides the habitat for the symbiotic bacterial community (Fig. 1C). This extracellular pattern—an inner, firmly adherent, layer with stratified organization devoid of bacteria, beneath an outer loose layer colonized by symbionts—is similar to the mammalian colon. This pattern of tissue layers may be a conserved principle in animal anatomy and points to the epithelium as a crucial regulator of microbial homeostasis (Schröder & Bosch, 2016). Conversely, the microbiota has a profound impact on both form and functioning of the epithelium. Observations on long-term germ-free animals indicate, that *Hydra*'s developmental pathways are dependent on the microbiota (J. Je & T.C.G. Bosch, unpubl.). Since this dependence includes transcription factor and signaling pathways that are highly conserved in the animal kingdom, this reliance on microbes points to two important principles: (1) that development in most, if not all, animals relies on interactions with microbes, and (2) that some conserved developmental pathways initially had components that were induced by microbes.

In *Hydra* both epithelial layers play different and independent roles in development and morphogenesis. Classic experiments using chimeric strains (Wanek & Campbell, 1982) indicated that the ectoderm regulates body morphology, while the endoderm controls body size (Wanek & Campbell, 1982). This decades-old observation is interesting because body size in *Hydra* is determined not only by internal regulatory processes, but also by environmental factors (Mortzfeld, Taubenheim, Klimovich, et al., 2019). Abiotic influences include temperature, which directly affects Wnt and TGF-beta signaling. The observation that germ-free polyps are considerably larger than conventionalized animals (He & Bosch, unpubl.) gives an interesting indication of the importance of the "microbiome" in size determination (Beard & Blaser, 2002).

The epithelial surface is densely colonized by a stable multi-species bacterial community (Fraune & Bosch, 2007). The *Hydra* host shapes the specific microbiome by means of the innate immune system and a rich repertoire of antimicrobial peptides (Franzenburg, Walter, Künzel, et al., 2013). The presence and structure of *Hydra*'s microbiota is critical for the tissue homeostasis and health of the polyps. Remarkably, each *Hydra* species supports long-term associations with a different set of bacteria, suggesting that the host imposes specific selection pressure onto its microbiome (Franzenburg et al., 2013; Fraune & Bosch, 2007). The findings reveal that epithelia and components of the innate immune system play an active role in selecting the inhabitant microbiota via a complex genetic network. This work has contributed to a paradigm shift in evolutionary immunology:

components of the innate immune system with its host-specific antimicrobial peptides appear to have evolved in early metazoans because of the need to control the resident beneficial microbes rather than to fight invasive pathogens (Bosch, 2014).

The *Hydra* model system also has provided insights of general significance with the discovery of the role of interactions between symbiotic bacteria in colonization resistance (Fraune, Anton-Erxleben, Augustin, et al., 2015). In previous studies we discovered that these early evolutionary divergent animals not only select their bacterial communities, but also select for viral communities in a species-specific manner (Bosch, Grasis, & Lachnit, 2015). Viruses are an important part of the *Hydra* holobiont by their influence on the species-specific microbiome (Li, Lachnit, Fraune, et al., 2017).

Although the epithelial cells of *Hydra* had been considered as prime regulators of the microbiome, recent data uncovered a previously underappreciated role of the nervous system with its rich repertoire of neuropeptides that participate in controlling resident beneficial microbes (Augustin, Schroeder, Murillo Rincon, et al., 2017). Recent findings also show that microbes affect the animal's behavior by directly interfering with neuronal receptors (Murillo-Rincon, Klimovich, Permoller, et al., 2017). These observations provide new insights into the original role of the nervous system, and suggest that it emerged to orchestrate multiple functions including host-microbiome interactions (Klimovich & Bosch, 2018). Below we illustrate that: (i) the presence and structure of this microbiota is critical for patterning processes and tissue homeostasis; (ii) the transcription factor FoxO provides a direct link between stem cell proliferation, tissue homeostasis, and the microbiota; and (iii) *Hydra*'s microbiome contributes to tumor formation.

2.1 FoxO is a key regulator of epithelial homeostasis and host-microbiome crosstalk

Besides its well-known conserved function as major tissue regulator and aging antagonist, the transcription factor FoxO modulates the innate immune system in various model organisms including mouse, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Hydra* spp. (reviewed in Mortzfeld & Bosch, 2017). In response to environmental or bacterial signals, FoxO can be shuttled between the transcriptionally inactive state in the cytoplasm and the active form in the nucleus. Binding of FoxO to conserved regulatory genomic regions can modulate the expression of genes involved with stem cell and immune functions (AMPs) and thus has a crucial impact on the aging process.

In *Hydra*, FoxO is regulated by symbiotic microbes, involving it in a multi-species set of interactions that regulates the aging and maintenance of the metaorganism. In *Hydra*, the microbiome is selectively assembled by a species-specific combination of AMPs that are predominantly expressed in epithelial cells (Franzenburg et al., 2013). FoxO-deficient *Hydra* polyps show, in addition to defects in stem cell maintenance (Boehm et al., 2012), a severe change of the immune status, and drastically altered expression of AMPs. Since FoxO-deficient polyps are impaired in selection for bacteria resembling the native microbiome, and more susceptible to colonization by foreign bacteria (Mortzfeld, Taubenheim, Fraune, et al., 2018), FoxO signaling not only plays a key role in the communication between host and microbiota, but also incorporates the evolutionarily conserved longevity factor FoxO into the metaorganism/holobiont concept (Fig. 3C).

2.2 WNT/β-catenin signaling is influenced by microbial colonization

Hydra has provided important insights into general principles of biological pattern formation. Regeneration and transplantation experiments performed as early as 1909 revealed that the tip of the *Hydra* head has organizer function (Browne, 1909), and the formation of the head organizer involves the canonical Wnt pathway (Broun, Gee, Reinhardt, & Bode, 2005; Gee, Hartig, Law, et al., 2010; Hobmayer, Rentzsch, Kuhn, et al., 2000). Treatment with alsterpaullone (ALP), a GSK3 inhibitor, results in increased activity of the head organizer system in the body column (Gee et al., 2010). Intriguingly, germ-free *Hydra* polyps are significantly more sensitive to ALP than control polyps (Taubenheim, Willoweit-Ohl, Knop, et al., 2020) (Fig. 1D). Germ free animals have four times more ectopic tentacles after ALP treatment compared to control animals. It therefore seems that the presence of the microbiota promotes the activity of the proliferation zone and inhibits terminal differentiation. Screening for genes affected by the bacterial colonizers resulted in the identification of four *Hydra* genes that were upregulated by bacterial colonizers and showed spatially and temporally regulated expression patterns (Taubenheim et al., 2020). Gene expression analyses led to the identification of a small, secreted protein, named Eco1a, being upregulated in the response to both bacterial colonizers and low temperatures. Loss-of-function studies showed that this novel microbe-dependent effector gene is indeed involved in the regulation of pattern formation and has an antagonistic function to Wnt signaling in *Hydra* (Taubenheim et al., 2020).

2.3 *Hydra*'s microbiome contributes to tumor formation

Development requires precise spatial and temporal activation of developmental signaling pathways so that cells do find their way into organized and "well behaved" structures. Cancer is primarily caused by the disruption of normal cellular functions through genetic, epigenetic, and paracrine changes. Once a tumor is initiated, however, complex interactions between cancer cells and their surrounding environment—known as the tumor microenvironment—can contribute to disease progression.

The recognition that certain bacterial and viral pathogens have long been considered as potential causes of cancer begs the question: does the healthy microbiome play a role in the development of cancer? Several years ago, Domazet-Lošo and colleagues used *Hydra* as phylogenetically ancient multicellular organism to demonstrate that in principle all multicellular animals can form tumors (Domazet-Lošo, Klimovich, Anokhin, et al., 2014). Cancer is now considered a legacy of transition to multicellularity early in the evolution of life. Using the experimental accessibility of the *Hydra* holobiont, recent research has demonstrated that after an environmental disruption of the normal bacterial community, the tissue of a polyp may be colonized by bacteria from the environment. The contact with already present microbes then leads to the production of bacterial factors that have a damaging effect on the *Hydra* cell structures, disturb tissue homeostasis and developmental pathways, and ultimately trigger tumor formation (Rathje, Mortzfeld, Hoeppner, et al., 2020).

Hydra develop cancer if a specific type of foreign bacteria from the phylum *Spirochaetes* becomes increasingly prevalent in the microbiome, and thereby disrupts the balance of the bacterial colonization in their tissue. Interestingly, these bacteria exert their harmful influence only in the presence of certain other bacteria from the genus *Pseudomonas*, which are part of the normal composition of the microbiome. The invading bacteria successfully colonize the host tissue only if the *Hydra* tissue has already been weakened by changing factors in the environment. These factors include a change in temperature, and the resulting change in microbial colonization. When they encounter each other, the bacteria modify their movement patterns and seek direct contact with one another. As a result, they also begin to express different genetic information and activate factors that have a pathogenic effect for the host organism. Due to these changes, the microbiota composition becomes drastically altered, which is followed by structural changes in the *Hydra* epithelial cells and ultimately leading to tumor formation. How these interactions occur at the molecular level, and

which specific biochemical mechanisms are involved in this form of cancer development, are the subject of currently ongoing investigations.

In light of these observations, the pathogenesis of cancer can be seen as a comprehensive interaction of genetic and environmental factors, including specific microbial influences. Since it was only the common presence of certain bacteria interacting with each other within a disturbed microbiome that enabled the formation of tumors, it is probably not a single malicious intruder, but the malfunction of the microbiome as a protective barrier for the body as a whole what can promote the development of cancer. These findings offer a promising perspective because the protective function of the microbiome could possibly be used in future: The microbial colonization of the body normally balances out and protects the organism against harmful influences, potentially even against carcinogenic influences. Future research will show whether this ability of the microbiome to maintain a healthy barrier, which protects the body from colonization by harmful microorganisms, may also be used for the prevention of cancer.

3. The binary light organ squid-vibrio model sheds light on basic mechanisms of symbiosis development

The association of the Hawaiian bobtail squid *Euprymna scolopes* and the luminous vibrio bacterium *Vibrio fischeri* has been studied for over 30 years (for companion reviews on host and symbiont see: Nyholm & McFall-Ngai, 2020 and Stabb, Visick, & Ruby, 2020 *Nature Reviews Microbiology*). At the time of this writing, 18 labs with a principal focus on the squid-vibrio system have opened in 14 states or US territories. This natural model symbiosis has been used to identify and characterize the conserved features of the chronic colonization of animal epithelia by gram-negative bacteria, perhaps the most common type of host-symbiont interaction across the animal kingdom. [The female of this species also has a consortial symbiosis, which has been studied over the last few years (Kerwin, Gromek, Suria, et al., 2019), that will not be discussed in detail here.]

Being a one-host/one microbial species association (Fig. 1), the squid-vibrio partnership is similar to the rhizobia-legume symbioses in plants; these relationships have provided unparalleled insights into conservation of symbiosis characters, particularly those shared in the development of alliances between animal and plant hosts and their microbes (Hirsch & McFall-Ngai, 2000). These binary associations offer high resolution for determination of the precise dialog between partners. They do not address

mechanisms of symbiosis with consortia of microbial species, but rather provide a window into the responses of small clones of bacteria associated with host cells, such as in the mammalian gut. Further, they provide low "noise" for studies focused upon determining the impact of the enormous symbiont strain variation that has been observed in a variety of symbiotic systems, including the squid-vibrio and human associations (Bongrand, Koch, Moriano-Gutierrez, et al., 2016; Koehler, Gaedeke, Thompson, et al., 2019; Yan, Nguyen, Franzosa, et al., 2020). A most critical advantage of binary systems is the opportunity to genetically manipulate all naturally occurring partners of the symbiosis and interpret the influence of these manipulations on symbiosis onset, development and persistence in the host. Such powerful methods have been available in the rhizobia-legume symbioses for over 25 years (for review see Roy, Liu, Nandety, et al., 2020), but, until recently, no animal models existed where we knew the detailed genetics of both partners. In the last few years, we've come to understand the genetic systems in both partners of the model system between the bean bug *Riptortus pedestris* and its bacterial partner *Burkholderia* sp. (Takeshita & Kikuchi, 2017), and researchers at the Marine Biological Laboratory (University of Chicago, Woods Hole, MA, USA) are analyzing the genetic systems of squids (Crawford, Diaz Quiroz, Koenig, et al., 2020), including the bobtail squid. These breakthroughs bring the power of genetic tools in all partners of the symbiosis to the animal world.

Several features specific to the squid-vibrio system make it an ideal candidate for the study of the developmental biology of symbiosis from ecology and evolution to physiology, biochemistry and molecular biology. *V. fischeri* is the only species that can colonize the light organ of *E. scolopes*. As such, the animals can be raised "aposymbiotically," i.e., exposed to the typical one million environmental bacteria per milliliter of seawater without *V. fischeri* present, and the organ will remain uncolonized. In addition, as noted in other cephalopods (Lutz, Ramírez-Puebla, Abbo, et al., 2019), male *E. scolopes* does not harbor bacteria in other regions of the body; strong antimicrobials keep the tissue microbe-free (Chen, Krasity, Peyer, et al., 2017). Further, several characters make this system ideal for study of development, including: (i) the bobtail-squid females mate and lay serial clutches of ~200 eggs/clutch in captivity throughout the year; as such, a facility with ~12 females and 8 male adults will yield ~50,000 juveniles/year for experiments with a large "n"; (ii) the embryonic period is ~20 days, and the juveniles hatch a dusk in large cohorts, which allows synchronization

of symbiont inoculation; (iii) juveniles average ~2 mm in mantle length, and the juvenile light organ is ~400 μm across, so the whole system is an ideal subject for confocal microscopy; (iv) first full colonization of the light-organ crypts, where the symbionts reside, takes place over ~12 h, so events can be visualized in real time; (v) successful symbiosis onset can be monitored non-invasively by monitoring symbiont light output from the animal with a photometer; and, (vi) because the "currency" of the symbiosis is light production for host camouflaging, the animal can be raised aposymbiotically through its life in the laboratory, i.e., the symbiosis is ecologically obligate, but, unlike symbioses that are nutritional or involved in tissue defense, the host is physiologically unharmed by the aposymbiotic state; as such, the impact of interactions with microbes during development can be characterized over the trajectory of host ontogeny, from symbiosis onset through maturation.

3.1 Embryonically-developed biophysical and biochemical features work in concert to achieve specificity of the association

V. fischeri cells, at a few hundred per mL of seawater, only represent a small fraction of the bacterial cells in the environment (Lee & Ruby, 1995). Evolution has selected biophysical and biochemical determinants in host and symbiont that promote efficient recruitment and selection of the specific symbiont cells. Following embryogenesis the juvenile host is poised to interact with the symbiont immediately upon hatching (Fig. 3A and B, upper and lower left). The tissues that give rise to the light organ form in the hindgut-ink sac complex. Invaginations on the surface form the six independent crypts, and swellings on the surface form the juvenile-specific ciliated fields that potentiate light-organ colonization (Montgomery & McFall-Ngai, 1993). Also during embryogenesis, the biochemistry of the organ is established that will promote engagement of *V. fischeri* at the exclusion of other bacteria in the environment; acidic mucus stores are laid down in the cells of the ciliated appendages, that contain a complex cocktail of antimicrobials (Davidson, Koropatnick, Kossmehl, et al., 2004; Heath-Heckman, Gillette, Augustin, et al., 2014; Kremer, Schwartzman, Augustin, et al., 2014; Troll, Bent, Pacquette, et al., 2010).

Shortly after hatching, the long cilia on the light-organ surface begin to metachronally beat (Fig. 3B, center; Nawroth, Guo, Koch, et al., 2017) and, within the first 0.5 h, the acidic mucus is shed in a non-specific response of the tissues to free cell-wall materials, or peptidoglycan (PGN), of environmental

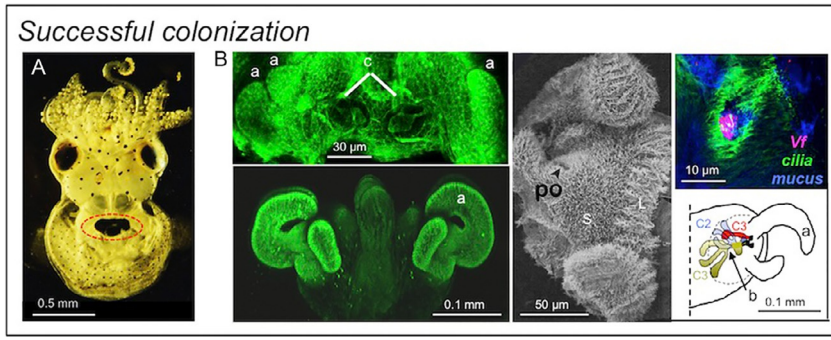

Fig. 3 Early development of the association. (A) Newly hatched *E. scolopes*; red circle, location of the nascent light organ in the mantle cavity. (B) *Left, upper*: a confocal image of the light organ about 2/3 of the way through embryogenesis; white lines indicate invaginated regions where the epithelium-lined crypts are forming. *Left, lower*: confocal image of the complex ciliated fields of the hatchling; a fully developed appendages on the light organ surface. *Middle*, a scanning-electron-microscope image of the ciliated surface, the metachronal waves can be seen along the long cilia (L) of the appendages and medial region of the ciliated field; surrounding the pores are short cilia (s) that randomly beat to mix biomolecules above the pores (po). *Right, upper*: a confocal image showing an aggregate of *V. fischeri* entering a pore on the organ surface. *Right, lower*: the three independent regions colonized on each side of the organ; after the bacteria enter the pores, *V. fischeri* cells travel through three biochemically and biophysically distinct regions over about 100 µm; at the medial portion of the biogeography of each of the three regions is a bottleneck (b) that allows, on average, a single *V. fischeri* cell to enter and grow out in one of the three crypts (C1, C2, C3). a, (left two images and right bottom image), superficial light organ appendages.

bacteria (Fig. 3B, upper right; Kremer et al., 2014; Nyholm, Deplancke, Gaskins, et al., 2002; Nyholm, Stabb, Ruby, et al., 2000). *V. fischeri* cells aggregate above the pores for minutes to hours, depending on the strain (Koehler et al., 2019; Nyholm et al., 2000); the vast majority of the research has been done with *V. fischeri* ES114, which is a strain that aggregates for about 3 h before entering host tissues. The subsequent discussion will be restricted to consideration of colonization by strain ES114.

Because many, if not most, bacteria in the seawater are around the size of *V. fischeri*, the action of the ciliated fields cannot be the only selection determinant. Detailed study of the system showed that, although in the absence of *V. fischeri* other gram-negative bacteria will aggregate on the hatchling organ surface, gram-positive bacteria do not aggregate (Koehler et al., 2019; Nyholm et al., 2000). Many of the antimicrobials shed with the mucus at hatching, including a peptidoglycan-recognition protein with amidase activity (EsPGRP2; Troll et al., 2010) and lysozyme (R. Augustin & M. McFall-Ngai,

unpublished data), are specific for gram-positives, which is likely to contribute to their absence in the aggregates. When *V. fischeri* is present, it becomes the competitive dominant over other gram-negative bacteria for that space through the aggregation process. Studies of the symbiont-cell behavior and host transcriptome at this time, showed that ~3 h, with a natural aggregate of ~5 *V. fischeri* cells, these cells attach to the cilia near the pores (Altura, Heath-Heckman, Gillette, et al., 2013); the host responds to the presence of this small number of *V. fischeri* cells, associating with only one or two host cells, with a detectable change in host gene expression across the ~10,000-cell hatchling light organ, a testament to the host sensitivity to its specific symbiont. Studies of the system provide evidence that this change has two effects: (i) an increase in the antimicrobials, which creates a "cocktail" that favors *V. fischeri*; and (ii) the breakdown of polymeric chitin, also in the mucus, into chitobiose (Kremer, Philipp, Carpentier, et al., 2013).

Genetic studies with *V. fischeri* have demonstrated that, to colonize normally, they must be motile (Aschtgen, Brennan, Nikolakakis, et al., 2019) and express genes that confer acid (Schwartzman, Lynch, Flores Ramos, et al., 2019) and nitric oxide tolerance (Dunn, Karr, Wang, et al., 2010; Wang, Dunn, Wilneff, et al., 2010), conditions more acute in the light-organ ducts (Davidson et al., 2004). Also while aggregating they express genes for chemotaxis to chitobiose, which is also more abundant in the ducts (Kremer et al., 2013). Thus, during the aggregation process the symbiont cells become primed to resist the stressful biochemical environment of the host tissues and to chemotax into the organ. After some residence in the aggregate, *V. fischeri* cells express a gene that mediates detachment from the cilia (Christensen, Marsden, Hodge-Hanson, et al., 2020), and then they move into host tissues (Fig. 3, upper right). Only *V. fischeri* can be found living in host tissues beyond the pores. Once inside one of the pores, an aggregate of *V. fischeri* cells begins a ~100-μm journey (Fig. 3, lower right; Essock-Burns, Bongrand, Goldman, et al., 2020) through a duct that opens into larger antechamber. The entrance to a narrow bottleneck is on the medial side of antechamber. This bottleneck has a critical function as a gatekeeper. Although many cells can make it to the bottleneck, on average a single *V. fischeri* cell enters a crypt and grows out to populate the space. This gatekeeper also serves to restrict *V. fischeri* to the regions of the crypts.

As mentioned, the above findings were the result of experiments with *V. fischeri* ES114. Studies of strain variation have shown efficiency of aggregation and colonization are highly strain dependent (Bongrand et al., 2016;

Koehler et al., 2019). These new data offer an exciting horizon for the study of variation in host development in response to colonization by different symbiont strains.

3.2 Symbiont molecules and light production induce early morphogenesis of the host organ

At about 12 h following first exposure to *V. fischeri* cells, the populations of symbionts have grown out to fill the crypt spaces. At this point, they begin to luminesce in response to the buildup of their homoserine-lactone molecules associated with quorum sensing (Lupp & Ruby, 2005). Concomitantly, the symbionts induce host morphogenesis (Fig. 4) (for review see McFall-Ngai, 2014). The most dramatic reflections of this phenomenon are the induction of hemocyte trafficking into and the widespread apoptosis of the superficial ciliated fields on the organ, which occurs several cell layers away from the colonizing symbiont. These events are triggered by microbe-associated molecular patterns (MAMPs) (Fig. 4A). Specifically, the morphogens are the lipid A of the lipopolysaccharide molecule (LPS) of the *V. fischeri* outer membrane and the peptidoglycan (PGN) fragments of the symbiont's cell wall; both LPS and PGN are bacterial molecules typically associated with perturbing homeostasis of animal cells. Shortly after the irreversible bacterial signaling of morphogenesis, the juvenile squid begins to "tame" the symbiont by detoxifying the MAMPs (Rader, Kremer, Apicella, et al., 2012; Troll et al., 2010) and also by attenuating the production of antimicrobials (Davidson et al., 2004). Although the phenomenon of bacterial MAMPs-induced morphogenesis was first discovered in the squid-vibrio system, these molecules also are now known to mediate microbiome-induced development and maturation in mammals (Bouskra, Brézillon, Bérard, et al., 2008; Rakoff-Nahoum, Paglino, Eslami-Varzaneh, et al., 2004).

In addition to driving morphogenesis of the surface epithelium, *V. fischeri* colonization of the organ induces development and maturation of the internal tissues (Fig. 4B; for review see McFall-Ngai, 2014). With actin rearrangements, the duct diameter decreases, as does the bottleneck (Essock-Burns et al., 2020), although the symbiont's driver of this phenotype remains unknown. In addition, the symbionts induce cell swelling of the crypt epithelial cells and an increase in microvillar density, which is mediated by lipid A (Heath-Heckman, Foster, Apicella, et al., 2016). The bottleneck closes down and only opens each day with the dawn venting of the symbionts into the surrounding seawater (see discussion below section on daily rhythms of the symbiosis); then, it closes down again within an hour

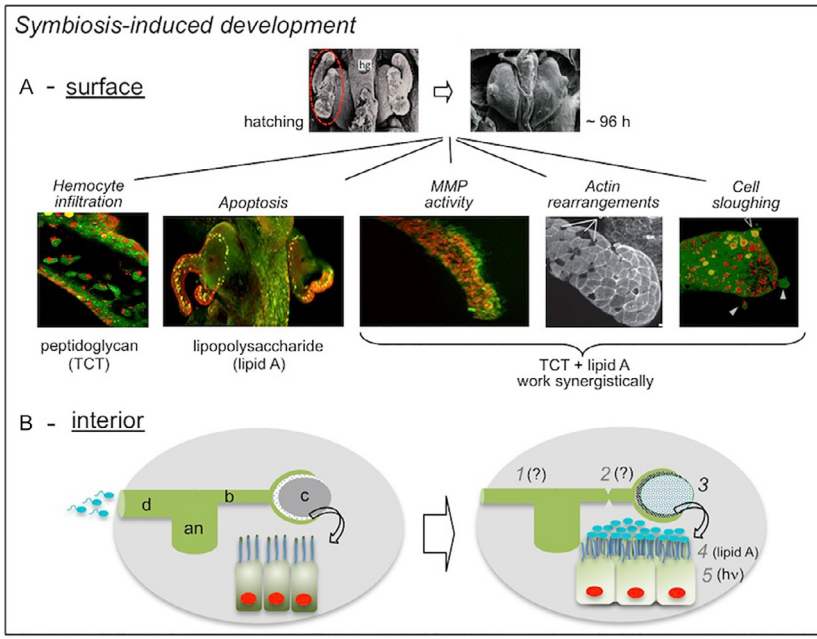

Fig. 4 Symbiont induced early development in the squid-vibrio symbiosis. (A) Colonization-induced loss of the superficial ciliated field that fosters colonization. Upper images: scanning electron micrographs of this process. Lower images: confocal images of the cellular events associated with morphogenesis and the symbiont MAMPs that are largely responsible. (B) Symbiont-induced changes in the light organ interior. Left, diagram showing the microbiogeography of the organ and the path of the symbionts to the crypts (c); arrow indicates an abstraction of the cells the line the crypt to show that they are columnar at hatching; an, antechamber, b, bottleneck, d, duct. Right, the five regions that undergo developmental change: 1, 2—duct and bottleneck respectively; symbiont inducers for these changes have not been identified; 3—the crypts become filled with a dense population of *V. fischeri* cells; 4—crypt epithelial cells change in microvillar density, due to exposure to symbiont lipid A; and 5—crypt epithelial cells swell, in response to symbiont luminescence, respectively.

after venting, restricting the regrowth to the crypt region. Taken together, the organ transforms from an anatomy and morphology that encourages symbiont recruitment to one that discourages subsequent colonization and tightly restricts *V. fischeri* cells to a specific region of the tissues.

It remains unknown how the presence of symbiont MAMPs, or other active symbiont molecules in the crypts, is communicated to the light organ surface to induce developmental events. The MAMPs are presented both as freely diffusing molecules and as constituents of outer membrane vesicles (OMVs) that are shed by *V. fischeri* (Aschtgen, Lynch, Koch, et al., 2016;

Koropatnick, Goodson, Heath-Heckman, & McFall-Ngai, 2014). These OMVs also carry other materials that are imported into the host cells, including symbiont non-coding RNAs, which change host gene expression, as well as proteins (Lynch, Schwartzman, & Bennett, 2019); and, some of the imported materials target the nucleolus and euchromatin (Cohen, Aschtgen, Lynch, et al., 2020). A current research focus explores which symbiont molecules in the OMVs are critical for normal developmental responses in the host.

One of the greatest drivers of symbiont-induced host development is not a biomolecule, but rather light production by the bacteria (for review see McFall-Ngai, Heath-Heckman, Gillette, et al., 2012), the principal "currency" of the association. In a *V. fischeri* mutant defective in light production (*delta-lux*), all of the above mentioned developmental events are delayed. The most conspicuous defect is the lack of cell swelling in *delta-lux* colonized animals. Comparing the 24-h transcriptomes between apo- and symbiotic organs, either colonized by wild-type or *delta-lux* strains (Moriano-Gutierrez, Koch, Bussan, et al., 2019), revealed that symbiont light production is the *principal* driver of colonization-induced change in light-organ gene expression. Interestingly, in this same study, it was discovered that the gene expression of remote tissues, specifically eyes and gills, was also influenced by colonization of the light organ; however, whereas gills had no difference in this phenotype by colonization with the *delta-lux* strain, all symbiont-induced gene expression in the eye was abrogated when the light organ was colonized with the *delta-lux* strain. These data, along with several other studies, have shown that the eyes and light organ are highly convergent in form and function, from their biochemistry and the control of their gene expression to their anatomical features.

3.3 Symbiosis persistence—The symbiosis is on a profound daily rhythm that undergoes maturation in the weeks following establishment of the partnership

Once a symbiosis is established, how is it maintained throughout the life of the host? In many symbioses, particularly those in which the microbial cells are extracellular, the symbiont cells usually continue to grow, so control of symbiont number is critical. Years ago, the squid-vibrio system reported the first daily rhythm on a bacterial symbiosis (Boettcher, Ruby, & McFall-Ngai, 1996). It has now been well established that the gut symbioses of mammals, including those of humans, are on a profound circadian rhythm, with strong evidence that the bacteria actually drive those rhythms

(for review see Zheng, Ratiner, & Elinav, 2020). The squid host is a night-active predator, burying in the sand during the day and emerging at night into the water column to forage for prey. Symbiont light production peaks at night (Boettcher et al., 1996), when the host uses the bacterial luminescence for counterillumination, a camouflaging behavior common in marine environments. This day-night behavior is controlled by the variation in oxygen provision to the symbiont by the host; as in all bioluminescence reactions, oxygen is required for bacterial luminescence. While the juveniles do not exhibit a strong daily rhythm of foraging/burying during the night/day as adults, this cycling of luminescence begins with the first day of colonization (Boettcher et al., 1996). Analysis of the underlying drivers of this behavior showed that the expression of the light-organ-specific clock gene, *escry1* (*E. scolopes* cryptochrome 1), is high when luminescence is high, i.e., at night. In contrast, another cry gene, *escry2*, shows highest expression during the day in the eye when environmental light is bright. While the *escry2* gene of the eye cycles independent of colonization state of the light organ, the *escry1* gene cycles only when the symbionts are present, and this cycling requires light production by the symbionts, i.e., colonization with mutants defective in light production does not induce cycling of *escry1*. Finally, experimental manipulation of the system showed that light alone, however, will not induce cycling of *escry1* in the light organ; the luminescence works in synergy with MAMPs. The other behavior that begins with symbiosis onset is the daily venting of ~90% of the symbiont population into the surrounding seawater. At this time, the microvilli are effaced, but the cells remain more polarized in the juvenile organ, whereas in the adults there is marked disruption of the crypt epithelial polarization.

Does the mature symbiosis differ in its rhythms and, if so, when does that change begin in host ontogeny? A decade ago, a study of the symbiosis showed a profound change in the transcriptomes over the day-night cycle of both the adult host central-core epithelium, which supports the symbiont population, and the symbionts cells in the crypts (Wier, Nyholm, Mandel, et al., 2010). In the hours before dawn, all recognizable host cytoskeleton transcripts are upregulated; and after dawn they are all down-regulated. Transmission electron microscopy revealed that, before dawn, the crypt cells are disrupted, with microvillar effacement, shortening or absence of the zonula occludens and other junctional complexes between cells and the apical surfaces are blebbing into the crypt spaces. TEM showed that in the hours after dawn, the cells completely repolarize, regaining their microvillar surfaces and cell junctions. Analyses of the symbiont transcriptomes revealed

that they respond to the presentation of host membranes by expressing genes associated with the use of membranes, by anaerobic respiration, as a nutrient source. Once this food source is exhausted, and the bacteria have regrown to fill the crypt spaces, they go into type of stationery phase called a "metabolically active growth stasis." The transcriptional profile of the adult organ tissues provided evidence that the host then begins to feed the *V. fischeri* cells another nutrient, chitin, which they metabolize by aerobic fermentation.

Recent studies of the system have demonstrated that early symbiosis does not have such profound cycling; only after 3–4 weeks following the onset of the symbiosis do the daily cycles take on this complexity. This period is concomitant with the onset of discrete host behaviors, i.e., the typical burying in the sand during the day and foraging at night. Further analysis of the juveniles with symbiont mutants defective in chitin utilization showed that juveniles do not require this nutrient source; these mutants are lost from the symbiosis at 3–4 weeks (Schwartzman, Koch, Heath-Heckman, et al., 2014). In adult animals, however, chitin-rich host hemocytes traffic to the crypt spaces at night releasing their chitin stores for the nocturnal aerobic fermentation by the symbionts. Thus, only the adult animal metabolism exhibits pronounced day-night cycles of anaerobic respiration and aerobic fermentation, respectively.

The low level of hemocytes in the light-organ crypts during the day and high levels at night are controlled by a highly conserved cytokine (Koch et al., 2020), Macrophage Migration Inhibitory Factor (MIF), whose gene expression and protein production in the light-organ epithelium oscillate, with high levels during the day and low levels at night. In addition, when MIF is low in the organ, the symbionts are producing fermentation products and releasing bacterial surface molecules that are chemoattractive to the hemocytes. Also important here is that the blood pigment of the host, hemocyanin, is brought to the crypts loaded with the oxygen. The fermentation of chitin drives the pH of the crypt space down, which causes the efficient offloading of oxygen from the hemocyanin by the classic Bohr effect, which enhances luminescence of the symbionts (Kremer et al., 2014). As such, the host controls the metabolism of the symbionts so that luminescence is fostered during the night, when the host is active in the water column. In response to the dawn light cue, as mentioned above, the host animals vent much of their symbiont population, and the cycle begins again.

Although daily rhythms on the mammalian gut symbioses are now well documented, possible changes over ontogeny or maturation of the rhythms

have not yet been studied. Intriguing, however, are the high levels of MIF that have been reported in the mammalian intestine, where it functions in concert with the microbiota to enhance barrier function (Manes, Shulzhenko, Nuccio, et al., 2017; Vujicic, Saksida, Despotovic, et al., 2018), but whether it oscillates on a daily rhythm or controls the well-known circadian rhythms of blood cell migration to the gut is not yet known. In the squid-vibrio system, this cytokine controls nutrient provision to the symbionts, but its high levels during the day may also enhance barrier function as in mammals. Thus, certain controls on daily rhythms of symbioses may also be conserved across the animal kingdom.

In summary, the high resolution of this binary symbiosis has provided a window into the conserved mechanisms underlying the establishment and maintenance of symbiotic associations along the apical surfaces of animal epithelia. The research on squid-vibrio model has shed light on how embryogenesis prepares the juvenile animal of a horizontally transmitted symbiosis to recruit specific bacteria from the environment each generation into a permanent association. In addition, it has described how the elements of the partners, previously associated with microbial pathogenesis (e.g., microbe-associated molecular patterns, pattern recognition receptors, antimicrobial peptides), are actually drivers of development. Further, the system has led the way in defining circadian rhythms as the mechanism that is developed in the symbiosis to maintain the association in homeostasis. Using this symbiosis, the community of biologists now studying this system will continue to provide insights into how microbes shape animal biology.

4. Conclusion

Biology is a complex science at every level. As such, it demands mechanisms by which to make sense of and find patterns in its diversity. Since the advent of next-generation sequencing, biologists have become increasingly aware that animals are typically complex assemblages of organisms, i.e., the host and its associated microbial partners. To understand their development, we will need to bring on all available models. In an insightful essay on the nature of models [https://sites.google.com/a/ncsu.edu/emily-griffiths/whatisamodel.pdf], Emily Griffiths posits that four types of models can be constructive: conceptual, such as a diagram; in silico, i.e., mathematical models; in vitro, e.g., cell culture or tissue/organ extracts; and, in vivo, such as the animal model systems for developmental biology that we have discussed here. Although our focus here has been on in vivo models, we believe that two approaches will be essential for making progress in our

understanding of development of the holobiont: (i) an exploitation and integration of all four of the above mentioned model types; and (ii) continued exploration into the diversity of animal symbioses and an expansion of the array of new model systems for this subdiscipline. This frontier is indeed vast and rich for the community of developmental biologists.

Acknowledgments

We thank Scott Gilbert for the opportunity to contribute to this volume and for critically reading the manuscript. M.M.-N. is currently funded by National Institutes of Health (NIH) R37 AI50661 (to M.M.-N. and E.G. Ruby); NIH, Center for Biomedical Research Excellence P20 GM125508 (to M.M.-N. and E.G. Ruby); NIH R01 OD11024 and GM135254 (to E.G. Ruby and M.M.-N.); and a grant from the Gordon and Betty Moore Foundation (to A. Briegel, U. Leiden, M.M.-N., E.G. Ruby and A. Septer, U. North Carolina Chapel Hill). This work was supported in part by grants from the Deutsche Forschungsgemeinschaft and the CRC 1182 ("Origin and Function of Metaorganisms") (to T.C.G.B.). T.C.G.B. appreciates support from the Canadian Institute for Advanced Research.

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