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# Animal development in the microbial world: Rethinking the conceptual framework

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## Abstract

Animals have evolved within the framework of the microbes and are constantly exposed to diverse microbiota. This dominance of the microbial world is forcing all fields of biology to question some of their most basic premises, with developmental biology being no exception. While animals under laboratory conditions can develop and live without microbes, they are far from normal, and would not survive under natural conditions, where their fitness would be strongly compromised. Since much of the undescribed biodiversity on Earth is microbial, any consideration of animal development in the absence of the recognition of microbes will be incomplete. Here, we show that animal development may never have been autonomous, rather it requires transient or persistent interactions with the microbial world. We propose that to formulate a comprehensive understanding of embryogenesis and post-embryonic development, we must recognize that symbiotic microbes provide important developmental signals and contribute in significant ways to phenotype production. This offers limitless opportunities for the field of developmental biology to expand.

## 1. Introduction

### What do we mean by “development”?

Classical animal developmental biology has a tradition of (or has focused upon) studying the process by which animals grow and develop through ontogeny. Within this construct, the main processes involved include regional specification, morphogenesis, cell differentiation, growth, and the overall control of timing, much of which occurs in the embryo, the starting point for all animal species (Gilbert 2001). As an animal develops, different combinations of genes are switched on giving the species different traits. Various factors can change the combination of genes that are switched on. Critical stages of embryonic development are defined by key epigenetic modifications including global demethylation of the early embryo and remodeling of 5´-methylcytosine throughout embryo development (Morgan et al., 2005). Such changes allow the species to adapt and survive depending on the circumstance.

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Developmental biologists have long been fascinated by this relationship between development and evolution and the fact that homologous genes often play similar roles during the development of very different organisms. In the last three decades we have learnt that the underlying regulatory machinery is ancient because many of the genes and physiological pathways that govern life are similar among all living organisms from bacteria to humans (Domazet-Loso et al, 2010; Cheatle and Pick, 2016). In that context evolutionary developmental biologists also realized that early diverging animals such as sponges and corals are much more complex and „vertebrate“ like than previously thought. It is clear that the origin and evolution of developmental novelties lies in developmental processes. The fundamental concern of evolutionary developmental biology (evo devo) is to understand how development is tweaked over time. How do developmental pathways evolve? What are the evolutionarily conserved or universal codes of development? And what processes of development create biological diversity?

These concepts beg the question whether nuclear DNA is the only source of developmental instructions. As pointed out by John Tyler Bonner (1974), each life cycle is governed by some immediate instructions from the genes, by many gene-initiated instructions that were given in previous life cycles, and by signals originating from outside the organism. All of these instructions taken together govern the development of a new embryo. Further, through development, an animal’s genotype is expressed as a phenotype, exposing genes to the action of natural selection. Thus, the evolution of animal development is sensitive to environmental factors.

Evolutionary developmental biology (evo-devo) contends that evolution occurs through changes in developmental processes. Ecological developmental biology (eco-devo) has shown that microbes are critically involved in developmental processes (McFall-Ngai 2002; Gilbert et al 2015; Bosch et al 2019). If both of these statements are true, then changes in the developmental functions of symbiotic microbes can cause changes in evolution. This yields the new science of ecological evolutionary biology, eco-evo-devo (Gilbert 2001; Gilbert and Epel, 2015; Skúlason et al 2019). The life cycles of hosts and their microbial symbionts are intimately integrated (Gilbert 2019).

Anticipating this change of perspective from the “evolution of phenotypes” toward an “ecology of ontogenies”, John Bonner (1965, 1974) proposed that the unit of evolutionary selection was the life cycle, and he recognized that signals for developmental change could come from the environment, not only from the genome. Moreover, Leigh van Valen remarked in the early 1970s that “A plausible argument could be made that evolution is the control of development by ecology” (VanValen, 1973, p 488). Studying these environmental factors and ecological forces have largely been restricted to analyses of developmental plasticity due to abiotic forces (e.g., environmental temperature, pressure, salinity, oxygen concentration) or the biotic forces of predation, crowding, and competition.

Very recent work, however, has revealed a new element in the ‘equation’: the microbial world. What, then, is the role of microbes in animal development? This chapter seeks to address that question by expanding the eco -devo perspective. To do so, it explores how a

number of different animal groups use microbial cues to orchestrate developmental processes and how animal hosts and microbes interact at the molecular level.

## What makes us rethink the classic field of development?

Development in animals, plants, and fungi is not just the product of one genome. On the contrary, the nuclear genome of an animal comprises a relatively small fraction of the organism's total genetic repertoire, and the majority of genes is provided by microbes. These microbes can be obtained vertically (from the host's parents) or horizontally (from the environment, other host species, or other members of the same species). The phenotype is a consequence of host-microbe interactions, and these multi-organismic associations fundamentally alter the host's evolution and ecology (Roughgarden et al 2017). Taking an integrated view, we therefore see the host–microbiome system as a holobiont (Rosenberg and Zilber-Rosenberg 2013), or a compound meta-organism consisting of the macroscopic host along with its all of its symbiotic microbes (Theis et al 2016; Morris, 2018). Indeed, the physiology and immunity of most animals has been shown to be an intimate entanglement of host and microbial interactions. Functional nervous systems, digestive systems, and immune systems are often the product of both the host and the microbes (Gilbert et al 2012; McFall Nagai et al 2013; Tauber 2017; Pradeu 2012). Important in the context of this review is the recognition that microbial populations and communities are integrated into animal developmental programs often over the entire trajectory of an animal's ontogeny.

This new perspective radically changes our conception of the 'Hierarchy of Life' (Figure 1), our concept of what an individual is, and our concept of life cycles. The multispecies developmental program of most animals results, not in a monogenomic "individual", but in a holobiont with tissues and organ systems that are created by the host genome and an organ system that comprises the interacting communities of microbes coevolved with the host. These microbial communities occur in discrete regions of organ systems across the landscape of the host "Bauplan" (body plan). Each animal is both an individual and biomes of multiple interacting ecosystems (Lee and Mazmanian 2010) We are teams. As such, the microbiome organ system enriches the individual animal with levels of the microbial hierarchy, from organism to populations and communities, rendering the metaorganism (or holobiont) a chimera of nested hierarchical levels.

Within this conceptual framework, the development of vertically transmitted symbionts occurs in tandem with host embryonic development. In horizontally acquired symbioses, metaorganism development is more sequential. In these latter cases, the embryo develops features that allow the animal to recognize and recruit the specific coevolved microbes; then, at birth or hatching, the microbiome organ system develops and integrates with the host organ systems. However, this is not to say that horizontally acquired microbes do not affect early development. In mice, for instance, the population of microbes in the pregnant dam's gut can affect the phenotype of the mice developing in utero (Kimura et al 2020).

To keep pace with the paradigm of animals as holobiont consortia constructed by the interactions of numerous microbes and their genomes, we need to construct a developmental biology and an evo-devo that recognizes microbial agency from fertilization, through embryogenesis, and throughout maturation.

## 2. The development of multicellularity across all domains

### 2.1. Autonomous and non-autonomous development of multicellularity in bacteria

Multicellularity is a developmental phenomenon, involving the regulation of mitosis and (often) the differentiation of parts within a whole. Complex multicellularity has arisen by various developmental strategies multiple times in both the prokaryotes and eukaryotes (Rokas, 2008; Lyons and Colter, 2015). While, to the authors' knowledge, no animals develop normally under natural conditions without interactions with the microbial world, many examples of autonomous development of multicellularity have been characterized in the prokaryotes. Mechanisms used to generate asymmetry in a predivisional/parental cell are well known in microbes. For example, cell division in the single-celled aquatic bacterium *Caulobacter crescentus* results in two daughter cells which have different fates – one becomes a "stalked" cell, resembling the parental cell; the other grows a flagellum, becoming a "swarmer" cell that, when mature, swims away. *C. crescentus* thus accomplishes a fundamental function that underlies all development: it generates daughter cells that differ from the parent cell (Zhou et al., 2019; Lasker et al 2020). Interestingly, *C. crescentus* development is obligate, i.e., it is on a perpetual 'clock' that drives the differentiation independently from environmental conditions.

Other bacterial phylotypes undergo development in a response to environmental conditions, most often resulting in the production of spores. The most classic case of a system of bacterial multicellularity is illustrated by the development of *Myxococcus xanthus*, a social predatory bacterium that hunts for prey cooperatively (Bretl and Kirby, 2016). Under conditions of starvation, large populations of *M. xanthus* cells coordinate to migrate into a mass that develops a stalk. That stalk elongates and eventually differentiates a fruiting body with spores that disperse and germinate when prey is again plentiful.

*Bacillus subtilus* also has another sort of development, specifically within a biofilm (Vlamakis et al 2013). Microbial biofilms comprise either populations of a single microbial species or are mixed species assemblages. Typically of the integrity the biofilm is held together by biomolecules exported by the biofilm members. Recent approaches using phylotranscriptomics revealed (Futo et al, 2020) that *B. subtilis* biofilm formation shares many properties of embryonic development with discrete reproducible stages, with the oldest genes being expressed first and the newest last. The study concludes that biofilm formation is a true multicellular developmental process, indeed one in which its "ontogeny" appears to be recapitulating "phylogeny."

Distinct spatial distribution patterns of cells are also observed in experimentally established biofilm communities of other species, and particular processes of their evolution, metabolic capabilities, and resistance toward antimicrobials have been revealed (Haagensen et al, 2015). In other words, some microbes seem to have developed all of the genetic architecture that is required for spatial-temporal controlled embryonic development. The underlying evolutionary and developmental processes of these communities remain elusive, but this topic represents a rich arena for future exploration of the origins of developmental mechanisms.

Although the examples of prokaryotic multicellularity described thus far occur through autonomous development, some species require more than one genotype. A striking example can be found in the two-species phototrophic consortium 'Chlorochromatium aggregatum' (Overmann, 2010). It consists of one central non-photosynthetic motile cell surrounded by ~15 associated cells of another species that are non-motile photosynthetic cells. The phototactic behavior of the consortium is the result of coordination between these cell types, with the photosynthetic cells sensing the light and the motile cells driving the group toward the light. The cell division of the developing consortium occurs in a highly coordinated manner to promote this 'division of labor' between the two species. The development of other highly structured multispecies biofilms among prokaryotic species have been reported, although most widely studied are those associated with an animal host. For example, the development and maintenance of 'corncob'-like and 'hedgehog'-like nine-taxon structures of the microbial plaques of the mammalian oral cavity are the result of highly orchestrated developmental programs (Mark Welch et al, 2016).

### 2.2. Development of multicellularity in eukaryotes may never have been autonomous, rather requiring transient or persistent interactions with the microbial world

Eukaryotic multicellularity has arisen more than 25 times (Grosberg and Strathmann 2007; Wegener Parfey et al., 2011), and it has been accomplished through two major strategies: aggregation of individual cells as in *Dictyostelium,* and division of a single cell (zygote or spore) as in animals, plants and fungi (Bonner, 1998). As Leo Buss (1987) emphasized in "The Evolution of Individuality", the very existence of integrated multicellular organisms is an outcome of evolutionary processes, not a starting condition. One major clade of unicellular eukaryotes of particular interest here are the Opisthokonta, the group that in evolution gave rise to the animal and fungal clades. Within the ophistokonts, the closest living relatives of animals have unambiguously been shown to be the choanoflagellates (King et al. 2008; Ruiz-Trillo et al. 2008; Shalchian-Tabrizi et al. 2008; Torruella et al. 2015). Choanoflagellates are a globally distributed group of marine and freshwater protozoans with a highly distinctive morphology characterized by a whip-like flagellum and a collar of shorter hairs, resembling the food-filtering "collar" cells that line the channels of sponges.

Research on marine choanoflagellates over the last decade has demonstrated that their life history and in particular their development and reproduction is closely linked with the presence of developmental signals originating in symbiotic bacteria. In *Salpingoeca rosetta*, the development of multicellular colonies is dependent on a novel sulfonolipid signaling molecule, RIF-1 (Rosette-Inducing Factor-1) from the bacterium *Algoriphagus* sp. (Alegado et al. 2012). This is of interest not only because choanoflagellates are the closest living relatives of animals but also because Bacteroidetes bacteria, of which *Algoriphagus* is a member, predominate the microbiomes of animal intestines. In addition to RIF-1, *Algoriphagus* also produces lipid activators, synergistic enhancers, and inhibitors that regulate rosette development (Woznica et al. 2017). Interestingly, in the life cycle of *S. rosetta* bacteria are not only needed as developmental cues to trigger colony formation but also for mating. *Vibrio fischeri* bacteria induce the full sexual cycle in *S. rosetta* by

producing a protein, a chondroitin lyase named EroS (Extracellular Regulator of Sex). This protein induces fusion and meiosis in the choanoflagellates.

Similarly, microbial communities associated with the surfaces of plants are essential for their growth and morphogenesis (Wichard, 2015). The morphogenetic factor thallusin, for example, is an amino acid derivative produced by the bacterium *Zobellia uliginosa*, that is sufficient to induce thallus development in the marine macroalgae *M. oxyspermum* and partially promote thallus development in Ulva species. Studies of *Ulva* have also shown that morphogenesis may require multiple bacterial molecules. On their own, bacteria belonging to *Cytophaga* and *Roseobacter* genera induce incomplete *Ulva mutabilis* development, promoting either cell division or thallus differentiation, respectively. Only the combined activities of these bacteria fully restore normal morphogenesis, showing that synergistic interactions are required at the molecular level (Matsuo et al., 2005; Spoerner et al. 2012).

Taken together, bacteria appear as master regulators of the life history of some choanoflagellate species and induce morphogenesis in marine algae indicating that certain bacteria groups have a high level of influence on the development of marine eukaryotes. Apparently, from the very beginnings, our animal ancestors were able to interpret developmental cues from diverse microbes.

## 3. Evolution of development in animals

### 3.1. Evolution of transmission mode – horizontal vs. vertical transmission

The initial stages of animal development rely on the production of male and female gametes that, upon fertilization, produce the single-cell zygote. In light of this reduction to a single cell, how do coevolved symbiotic systems ensure persistence of the host-microbe association between generations? While a spectrum of transmission modes occurs cross the animal kingdom, most holobionts maintain their integrity between generations by one of two mechanisms - horizontal (environmental) or vertical (transovarian) transmission, which present very different developmental challenges for maintenance of the holobiont (McFall-Ngai, 2002; Bright and Bulgheresi 2010). In a horizontally transmitted symbiosis, the juvenile host recruits its specific symbionts from the surrounding habitat with each generation. As such, the microbial partners are not present to interact directly with host cells during embryogenesis (although, for indirect agency, see Kimura et al 2020).

However, evolution has selected for determinants developed during embryogenesis that allow the host to engage and recognize the coevolved, i.e., specific, symbionts, on hatching or birth. In these associations, the bacterial partners occur most often as extracellular consortia colonizing polarized epithelia, such as in the vertebrate alimentary canal (Sharpton, 2018; Song et al., 2020), the surface of *Hydra* (Schröder and Bosch, 2016) and the accessory nidamental gland of various squid species (Kerwin et al., 2019). However, some monospecific and/or intracellular alliances that exhibit environmental transmission do occur [e.g., the squid–vibrio mutualism (McFall-Ngai and Ruby, 1991) and the associations between vent tube worms and their sulfur-oxidizing bacterial symbionts (Nussbaumer et al., 2006). Typically following initial establishment of a horizontally transmitted symbiosis, characters are developed post-embryonically that promote symbiotic persistence. For

example, in systems as divergent as the squid-vibrio association and the ruminant microbial consortia, which have conspicuous anatomical features associated with symbiont recruitment, the juvenile specific morphology that facilitates horizontal transmission undergoes dramatic morphogenesis following symbiosis onset (see Sections 3.3.4 and 6.2). More subtle changes can also be observed, for example in the maturation of the gut-associated lymphoid tissue mammals, which requires exposure to Gram-negative symbionts (Bouskra et al., 2008).

Resident bacteria also play important roles in the maturation of the zebrafish intestine, including promoting intestinal epithelial cell proliferation and recruiting innate immune cells in the gut (Bates et al 2006; Cheesman et al 2011; Stephens et al 2016). These complex maturation and persistence processes mediate the mechanisms to maintain specificity, control of community diversity, prevention of symbiont loss or overgrowth, and finally a seeding of the environment with symbionts for the next generation.

In vertically transmitted symbioses, bacterial partners are carried in or on the eggs by the female parent (Douglas 2014). These types of associations are widespread, particular among the insects and certain mollusk clades (Cary, 1994) e.g., at deep-sea vent and seep environments), and restricted to the invertebrates. Rather than being superficial, the bacterial partners in such symbioses are most often intracellular constituents of organs located deep in the body cavity. While these alliances are difficult to study experimentally, they are not only very common but they are also often ecologically and economically very important associations and have become the subjects of a rich literature. Because the symbionts are provided in or on the egg, the microbial partners are incorporated into the host's embryonic program. As such, evolutionary selection pressure has fostered mechanisms for targeting of the symbionts to specific tissues/organs during embryogenesis and restricting the growth of the symbionts to these areas. Similar to horizontally transmitted symbioses, features are developed during maturation that promote a stable and controlled symbiosis.

### 3.2. The generation of two or more germ layers and the origin of gastrulation as major milestones in animal evolution

Perhaps the most common types of interactions that eumetazoans have with microbes occur at the apical surfaces of the epithelia, either along the body surface or along the gut. Below we consider the evolutionary and developmental origin of these tissues and our current knowledge concerning the role of microbes as potential drivers of these processes.

**The endoderm as the first germ layer to evolve—**According to the germ layer hypothesis (v Baer 1828; Remak 1850), all tissues and organs in our body derive from one of three germ layers that are established during early embryogenesis. Ancestral metazoans such as the Cnidaria have only two cell layers, an ectoderm and an endoderm. Following Thomas Henry Huxley (1871), the two layers of adult Cnidaria are homologous to two of the germ layers in vertebrate embryos - ectoderm and endoderm, i.e., they are conserved across the eumetazoa. Recent studies of the temporal and spatial components of developmental gene expression in several species (Hashimshony et al., 2015; Yanai 2018) show that the mesoderm of the Bilateria was the last germ layer to evolve because the gene expression

program of the mesoderm was found to be induced after those of the ectoderm and endoderm. Interestingly, the endoderm expression programs (and the endodermally active genes, themselves) preceded the ectodermal genes in nematodes (e.g. *Caenorhabditis elegans*), frogs (e.g. *Xenopus tropicalis*), cnidarians (e.g. *Nematostella vectensis*), and sponge (e.g. *Amphimedon queenslandica*) embryogenesis. Therefore, it may well be that the endoderm program dates back to the origin of multicellularity, while the ectoderm originated as a secondary germ layer.

**Epithelia in "pre-bilaterians" are in close contact with colonizing microbes—** Work in metazoan animal models such as the freshwater polyp *Hydra* has shown that much of the behavior of the epithelial layers is impacted by the interactions with colonizing microbes. Both, ecto- and endodermal epithelial cells produce a rich repertoire of antimicrobial peptides which regulate the microbiome (Franzenburg et al., 2013). Conversely, as we will describe later (chapter 5.1., Figure 3 C), the microbiota has a profound impact on both form and functioning of the epithelium. Observations on long-term germ free pre-bilaterian animals such as *Hydra* indicate that developmental pathways are influenced by the microbiota (He and Bosch, unpubl.). Since this includes transcription factors and signaling pathways that are highly conserved in the animal kingdom, it may well be that development in all animals is relying on an interaction with microbes in a specific manner.

**Thoughts about microbial influences on the origin of gastrulation and the evolution of the gut—**An important step in animal embryology is the laying down of a second and a third layer of cells so that the body has an outside of cells (ectoderm), an inside (endoderm), and a middle one (mesoderm). This process begins with gastrulation, which can occur either by invagination, by the ingression of cells from the outer blastula layer to fill the blastula cavity. In both cases, gastrulation leads to the formation of the archenteron, which will give rise to the endoderm and the mesoderm. Whatever drove the evolutionary pressure for gastrulation was certainly some abiotic or biotic environmental feature. In his gastraea theory, Ernst Haeckel (1874) imagined a gastrula that was like a planula larva on a biofilm, where an intrusion is created that produces a restricted, nutritive, and relatively stable environment. Such an environment is the ideal situation for the first interactions with the microbial world, particularly with the recognition that benthic biofilms are typically bacterial. Recent research has indicated that one the strongest and most widely shared features across the animal kingdom is the carriage of microbes the gut (Brody 2020; Moeller and Sanders, 2020). It is likely that these interactions had a role in driving the form and function of the gut over evolutionary time and are also likely to do so within the life history of an individual animal.

The ruminants offer a striking example of the role of microbes in the evolutionary selection for a complex gut (Chiu and Gilbert 2020). Herbivory comes late into evolutionary history. Vertebrates, for instance (and most herbivorous insects) have no gene in their genome encoding enzymes capable of digesting cellulose, hemicellulose, or pectins. This capacity is given these animals by their bacterial symbionts. Herbivores evolved as complex associations between animal and symbiotic microbes. Perhaps the most successful of these

associations can be seen in ruminants, whose taxon is defined by this portion of their stomach (Hofmann, 1989). The rumen is a complex, dynamic ecosystem composed of mainly anaerobic bacteria, protozoa, anaerobic fungi, methanogenic archaea and phages (Jami et al., 2013); this fermenter produces volatile fatty acids (VFAs) that provide ~70% of the cow's daily energy requirement from their fiber-rich diets (Yeoman and Bryan, 2014). However, the calf is born functionally monogastric, without the fully developed rumen, and the rumen remains non-functional and underdeveloped until weaning. Once it gets fed grain and grass, anatomical alterations allow the fiber-rich diet to enter the immature rumen (Jami et al., 2013). The bacteria in the rumen, which had migrated there during the first week of the calf's life, can now breakdown the grain into short-chained fatty acids, including butyrate. The butyrate is the developmental signal for the growth and differentiation of the rumen (Baldwin and Connor, 2017; Sander et al., 1959). The regulation of rumen development is thought to be achieved through changes in DNA methylation and microRNA production (Li et al 2019; Malmuthuge et al 2019). By three months, the rumen comprises about 85% of the calf's stomach volume. Thus, through developmental symbiosis, the gut bacteria construct their own niche, the rumen, which will then perform the nutritive symbiosis that will enable the cow to survive. Here, developmental symbiosis, sympoiesis, has enabled herbivory--an entire new evolutionary trajectory.

The evolution of herbivory in both invertebrates and vertebrates depended on the interactions between gut microbes and the gut epithelia (Gilbert 2020). Bacterial symbionts not only affect the development and evolution of the vertebrate gut system, but they may also be the reason that some animal hosts do not develop a gut system at all. More than 100 species of gutless worms have been found in marine sediments around the world, and they have formed symbiotic relationships with bacteria that provide them with nutrition and have enabled them to colonize nutrient-poor environments. The symbionts are so efficient at feeding their hosts and recycling their waste compounds that the worms have completely reduced their digestive and excretory systems. In one species, the well studied vent tubeworm *Riftia pachyptila* (Felbeck, 1981; Nussbaumer et al., 2006) the juvenile animal starts with a gut. However, early in development the planktonic form of the bacterial symbiont bores into the body wall of the host and takes up residence and proliferates in the regions of the body that will become the trophosome. The associations between gutless marine worms and their bacterial symbiont communities are ideal model systems for the study of beneficial animal-microbe symbioses (Hinzke et al 2019; Jäckle et al 2019).

## 4. The influence of microbes on development through ontogeny

### 4.1. The role of bacteria in developmental programs of animals with complex life-history strategies

As mentioned above, the formation of biofilms is a common behavior among the prokaryotes, either in clones of an individual genotype or as mixed species assemblages. Animals have taken advantage of this microbial behavior under a variety of circumstances. Perhaps the most widespread and ecologically critical biofilms are those created on the benthic substrates of marine environments. Such biofilms are drivers of the settlement and metamorphosis of marine invertebrate larvae. Planktonic larvae of a wide variety of taxa in

at least 10 phyla, including sponges, and sessile cnidarians, annelids, mollusks and chordates species, require cues from specific bacterial species in biofilms to settle and metamorphose in a particular spot on the substrate (Hadfield, 2011).

It has become increasingly clear that this phenomenon shapes the benthic ecology of the world's oceans. However, while invertebrate biologists had long known that larvae do not settle randomly, because of the historical gulf between the fields of macro- and microbiology, it wasn't until the 1970s that the first characterization of bacterial biofilms as inducers of larval settlement was published (for review see Hadfield and Paul 2001).

The developmental trajectory of the serpulid polychaete *Hydroides elegans*, has been under intense study for more than 25 years and offers a striking example of how bacterial biofilms influence animal development (Freckelton et al., 2017). Early studies confirmed that the presence of bacteria within biofilms was essential for settlement and metamorphosis of *H. elegans*, and they demonstrated that bacteria known to be inductive often represent a very small fraction of the bacteria within a given biofilm. Further, within a bacteria species, there is strain specificity to the response. For example, the bacterial species *Pseudoalteromonas luteoviolacea* is a potent inducer of *H. elegans* settlement and metamorphosis, but not all strains of the species are inductive. Further, this triggering of development in *H. elegans*, as with other invertebrate larvae, can occur by exposure to several different bacteria in the biofilm. The bacteria *P. luteoviolacea* combines hundreds of phage-tail-like elements to make complex structures effectively induce the settlement and metamorphosis of *H. elegans*. In another inductive bacterium, *Cellulophaga lytica*, the outer membrane lipopolysaccharide of this species appears to be the active inducer of *H. elegans* development (Freckelton et al., 2020). Clearly an entire research horizon has been presented by these new and exciting findings in the developmental biology of marine invertebrates.

The above-described phenomenon of bacterial biofilm-induced development represents a situation where animal-bacterial interactions are essential, but they are not symbiotic systems where the organisms are living together (Freckelton and Nedved, 2020). Some symbiotic systems do, however, require bacterial biofilm formation during development. For example, in the onset of symbiosis in the bobtail squid, candidate symbiont microbes aggregate in biofilms above the sites of colonization. For this behavior, the cells of the symbiont, *Vibrio fischeri*, must export an exopolysaccharide that promotes cell-cell interaction during aggregation; mutants in this behavior do not colonize the host animal (Shibata et al., 2012). In the more complex mammalian systems, biofilms of the oral cavity and gut are recognized, but the determination of their positive and negative benefits in these situations is far from resolved. Further, the extent to which biofilm formation is an important element of development in terrestrial animals, i.e., along the mucosal surfaces, has not been widely studied as yet.

Microbial influences on the developmental programs of other animals with complex life history strategies are also being explored. Many marine invertebrates have multiple larval stages, but the microbiomes of only a few of these species have been examined. A recent laboratory study of the developmental stages of the sea urchin *Strongylocentrotus purpuratus* revealed that bacteria occur with the zygote, embryo, and early larval stages, but the largest

concentration of bacteria is recruited after feeding begins and localizes to the gut lumen (Schuh et al., 2020). Future work will need to both confirm this in the field and to determine which subset of these microbes are stable symbionts vs. transient microbial 'tourists', e.g., passing through with the food (Carrier and Reitzel, 2019; Carrier et al., 2019). Nonetheless, bacterial exposure in the early development of *S. purpuratus* is critical for the induction of normal developmental changes in morphology and in the maturation of the immune system. Specifically, the normal growth of the animal and its ability to resist pathogens are compromised in animals without the exposure to the dense populations of microbes typical to their seawater environment (Schuh et al., 2020). While the study of the impact of symbiosis on complex life history strategies is in its infancy in marine invertebrates, this topic has been extensive studied in insects for many decades (for review see, Hammer and Moran, 2019). As mentioned above, many insects have vertical transmission of symbionts. Whether the insect is hemi- or holometabolous can strongly impact the symbiosis itself in a variety of ways and can be impacted by the symbiosis, and new molecular tools are providing opportunities to define the mechanisms underlying these impacts. For example, a recent study of a weevil *Sitophilus oryzae* provides a striking example (Maire et al., 2020). These insects are holometabolous and the harbor symbiont *Sodalis pierantonius* in organs called bacteriomes. In this system, the bacteriome of the larvae, which contains the symbiont-containing bacteriocyte cells, dissociates. The bacteriocytes change gene expression and migrate through the tissues to the site where the symbionts will reside in the adult animals. Simultaneously, the symbionts change gene expression to allow them to invade host stems cells, move into the nuclei of these cells and induce bacteriocyte development to form the adult bacteriome. The host and symbionts have complex cellular and molecular dialogues that ensures the provision of the symbionts to the next life stage and the development of the productive association in a precise location.

## 4.2. Host-microbe interactions drive conserved developmental programs

**4.2.1. Symbionts educate the immune system—**Over the past few years, the field of immunology has been revolutionized by the growing understanding of the fundamental role of the microbiota in the induction, education, and function of the mammalian immune system (Belkaid and Hand, 2014). Studies in mice have covered the critical role of colonization by maternal microbes in early life for normal immune development, including innate immune cell production, antibody production, immune regulatory functions, epithelial barrier integrity, and even tryptophan and nucleotide metabolism in offspring. As the early-life microbiota is seeded by passage through the birth canal, the education of the immunity in offspring is critically dependent on the maternal microbiota. In addition, the maternal microbiome can also have an impact on disease development later in life. It seems that an altered microbiota can increase intestinal permeability and thus promote the development of a proinflammatory milieu that stimulates beta-cell autoimmunity in the predisposed host. For example, differences in human milk oligosaccharide-utilizing bacteria provide a route to differences in immune education that may predispose the body to immune and metabolic related conditions such as diabetes (Garidou et al., 2015); an altered gut microbiota during the weaning period (due to antibiotics, high fat diet etc) may hinder adequate development of host immune responses, which leads to increased susceptibility to immune-related diseases such as allergy in later life. Interestingly, microbial exposure after weaning does not

alter the development of immune responses, highlighting the critical window of weaning for preventing immunopathologies in adult life.

Obviously we are witnessing a paradigm shift in immunology: symbiotic microbes are essential for the development of the immune system; and conversely, immune systems control the symbiotic microbiota in addition to defending against invasive pathogens. Immunity is not just a host function. It is a holobiont function (Pradeu 2012; Gilbert and Tauber, 2016; Chiu et al 2017; Tauber 2017). This is true for the adaptive immune system where there is increasing support for the view (McFall-Ngai 2007) that a memory-based immune system may have evolved because of the need to recognize and manage complex communities of beneficial microbes. And this seems also true for the innate immune system with its host-specific antimicrobial peptides and rich repertoire of pattern recognition receptors, which control the resident beneficial microbes rather than invasive pathogens (Bosch 2014).

**4.2.2. Symbionts and neurogenesis—**Similar to the immune system, the development and evolution of the nervous system must be considered in the context of host-microbe interactions. Studies of germ-free animals or animals treated with broad-spectrum antibiotics show that specific microbiota can impact central nervous system (CNS) physiology and neurochemistry (Sharon et al 2016). GF mice that are devoid of associated microflora exhibit neurological deficiencies in learning, memory, recognition, and emotional behaviors (Gareau 2014; Foster et al 2017). Proliferation of neurons in the dorsal hippocampus is greater in GF mice than in conventional mice. However, post-weaning exposure of GF mice to microbial clones did not influence neurogenesis, suggesting that neuronal growth is stimulated by microbiota at an early stage (Ogbonnaya et al. 2015). The connection between microbiota and hippocampal neuronal generation is further strengthened by the findings that deficient neurogenesis can be counteracted by a probiotic combination of specific bacterial strains (Ait- Belgnaoui et al. 2014; Möhle et al. 2016).

While it was known for some time that NF-κB signaling participates in microbiota-neuron axis, recent studies indicate that microbiota disturbance leads to increased NF-κB activation and TNF-α expression with induced memory impairment in animal models, and the restoration of microbiota composition alleviates neuroinflammation in hippocampus and ameliorates relevant symptoms (Jang et al. 2018; Ma et al 2019; Sarkis Mazmanian et al).

Similar observations on the role of microbiota in training the nervous system were made in the enteric nervous system (ENS). Germ-free mice show an abnormal proportion of calbindin and nitrergic myenteric neurons, suggesting that the microbiota influences the maturation of intestinal neural networks (De Palma et al 2014; McVey Neufeld et al 2015; McVey Neufeld et al 2016). The functional activity of the ENS can be regulated by the microbiota either directly through Toll-like receptors (Brun et al 2013) or by inducing the secretion of BMP2 by macrophages (Müller et al 2014). Recent studies show that the immature enteric nervous system of germ-free mice can be normalized upon colonization with a normal microbiota (De Vadder et al 2018); and that the gut microbiota can induce maturation of the adult ENS through release of 5-HT and activation of 5-HT4 receptors (De Vadder et al 2018). From an evolutionary point of view, the discovery of an interaction of

microbes with the mammalian CNS and also ENS comes as no surprise. Functional transcriptomic data in *Hydra* indicate very clearly that nervous system evolved to sense and communicate via “antimicrobial peptides” (AMPs)with the microbes (Klimovich and Bosch, 2018). Indeed, prior to the evolution of the mesoderm, neurons seem to have evolved as immune cells (Klimovich et al 2020) to control the symbiotic microbiota. And conversely, it looks like since the beginning of animal evolution that microbes directly affect the functional activity of the nervous system in *Hydra* by controlling spontaneous body contractions. The hypothesis that gut bacteria are integral contributors to development and function of the nervous system (Sharon et al., 2016) and that a “gut microbiota-brain axis” exists is getting support from both experimental and clinical sources (Heitz et al2011; Sharon et al 2019; Sampson and Mazmanian 2015; Cryan et al 2019; Desbonnet et al 2014; Hsiao et al 2013.)

#### 4.2.3. Gut microbiota affects juvenile development, growth, and maturation—

The first evidence that the microbiota directly contributed to developmental processes came from observations revealing that bacteria provide developmental signals to the intestinal epithelia. Bacteria-induced expression of mammalian genes was first demonstrated by Umesaki (1984; see also Bry et al 1996) who noticed that a particular fucosyl transferase enzyme characteristic of mouse intestinal villi was induced by bacteria. Other studies have shown that the intestines of germ-free mice can initiate, but not complete, their differentiation. Germ-free mice have smaller intestines, with a paucity of enteroendocrine and goblet cells (Bates et al., 2006). Microbial colonization is accompanied by profound changes in the intestinal transcriptional programs (Rakouff- Nahoum et al., 2015). Moreover, microbiota also play a key role in constructing the microvascular network (Stappenbeck et al 2002). Here, it appears that bacteria such as Bacteroides thetaiotamicron are inducing the intestinal Paneth cells to activate the genes encoding Angiogenin-4. When Angiogenin-4 is secreted by the cells, it instructs the mesoderm surrounding the gut to form capillaries (Hooper et al 2001; Rhee et al 2004).

Mammals aren’t the only animals whose development of gut and immune system depend on microbial symbionts. By tagging bacteria with a fluorescent molecule to observe how and when they take up residence in the developing fish, University of Oregon scientists have discovered that bacteria colonize the immature gut soon after the larvae hatches, and before the animal is fully mature (Bates et al 2006). In the absence of microbiota, the zebrafish gut is arrested in specific aspects of differentiation, and the animals have trouble absorbing nutrients. All these defects can be reversed by the introduction of bacteria later in development. In zebrafish, microbial symbionts use the beta-catenin signaling pathway to initiate cell division in the intestinal stem cells (Rawls et al 2004).

Extensive evidence has also indicated that symbiotic microbes are critical to providing numerous functions in developing insects. As illustrated clearly by comparing germ-free and conventionalized *Drosophila* larvae cultured on protein-poor food, gut microbiota can contribute to the host juvenile development and maturation by positively impact insulin/ IGF-1 and steroid hormone signaling (Shin et al., 2011; Storelli et al 2011). While conventional larvae grow more slowly than on protein-rich food because of a decrease in TOR signaling and the consequent disarray in the insulin and steroid hormonal signaling

(Boulan et al 2015), germ-free larvae are much more delayed. *Wolbachia* bacteria accomplish numerous functions--from immunity to sex determination --in several species of insects (see Gilbert and Epel 2015), and in the nematode *Brugia malayi*, *Wolbachia* bacteria are responsible for the correct anterior-posterior polarity of the second mitotic division (Landmann et al 2014.)

Microbes, therefore, must be considered as an integral part of development. In comparing the zebrafish gut microbe interactions to those of mice, some responses appear conserved across millions of years of evolution (Rawls et al 2006), indicating that animals may possess a conserved program of interactions with the microbes with which they have coevolved.

## 5. Conclusions, opportunities and future perspectives: A paradigm shift and a new way of exploring development

Developmental biology has advanced remarkably and substantially enough for scientists to be confident for the first time that some aspects of development in some organisms are understood at the molecular level. Protein components are identified, their functions in developmental processes are known, and the time and place in the embryo of expression of the genes encoding them are known. Developmental biology however needs a 21$^{st}$ century upgrade that incorporates the impacts of the microbial world. Within this arena, many expansive vistas of research are presented that promise to drive developmental biology to the next levels of enquiry. This sampling of the open questions, some expansive and some more focused, includes:

- How do the interactions with the microbial world influence the developmental biology of an animal, from its molecular form and function to its ecological position within the biosphere?
- How does animal development ensure the deployment of cellular and molecular mechanisms for the integration of microbial influences across the holobiont?
- How does embryogenesis and postembryonic development promote microbiome-mediated communication across the body?
- How is symbiosis reflected in the host and symbiont genes that are expressed throughout the trajectory of development; does some portion of these genes designated as ‘hypothetical‘ encode the genes that drive formation of the holobiont? What portion is borrowed, or the result of evolutionary tinkering, and what portion is unique to that type of symbiotic organ, i.e., among the orphan genes of that organism?
- How will developmental biology of animals with their microbiomes be affected by climate change? How do environmental perturbations (e.g., changes in temperature, environmental pH) affect the normal development of the holobiont?
- Will disruption of symbiosis drive extinctions of the host and/or microbial partners?

- What role do animal-microbe interactions in development (e.g., metamorphosis by particular biofilm communities) drive invasions?
- How has the development of a symbiosis been modified during radiation into new habitats or niches (e.g., in the water-to-land transition)?
- To what extent does phenotype variation across species depends on species-specific microbiota? Since it is known that at least some animal species acquire and maintain their species-specific microbiota, do in turn microbiota contribute to phenotypes that are unique to each species?

Some of these questions are already under study in laboratories around the globe, but a more focused effort is required. The evidence is now irrefutable – the developmental biology of animals is in the Anthropocene, and has been over evolutionary time, shaped by interactions with the microbial world. As such, to be effective and full contributors to the field of biology, animal developmental biologists must switch from an isolated view of a discipline to one that includes this dimension.

In conclusion, this chapter sought to explore the current state of developmental biology in the microbiome era. The stunning range of opportunities now available for an integrative evolutionary developmental biology so that we have an excellent chance to move toward a future comprehensive understanding of animal development.

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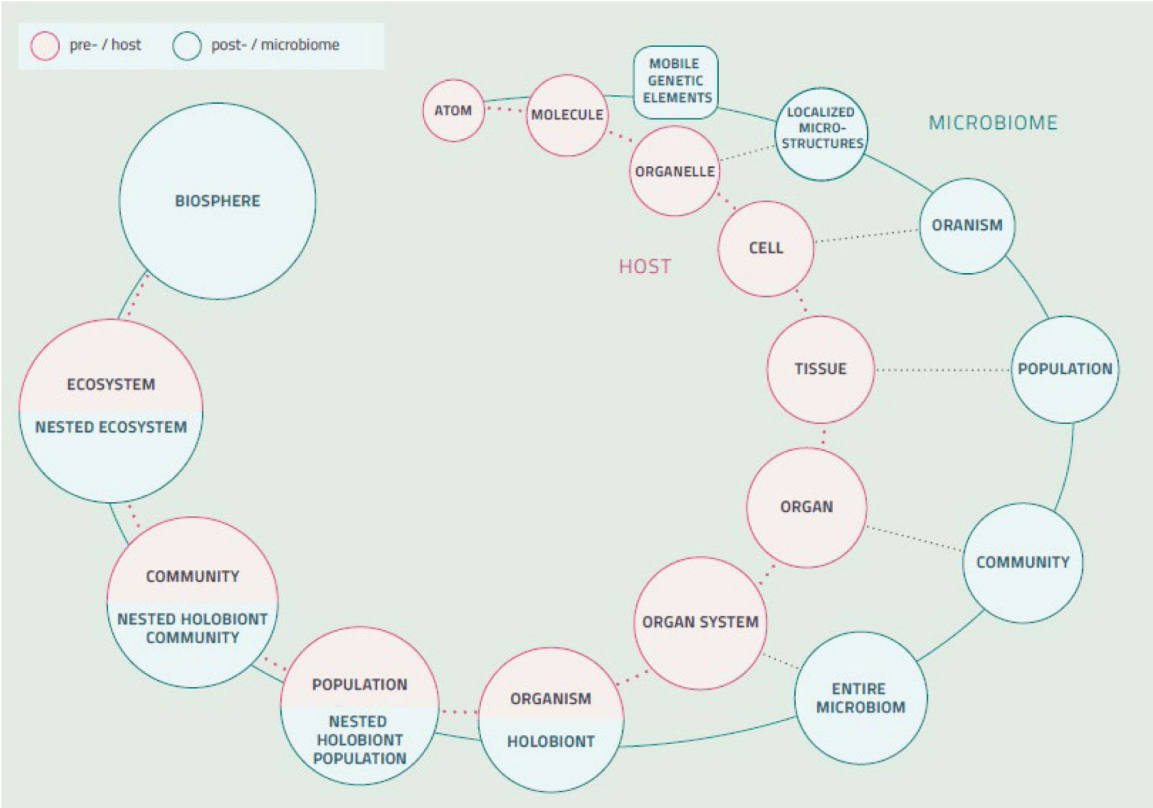


**Figure 1.**

Bosch and McFall-Ngai

Incorporating coevolved symbioses into the hierarchy of life. The recognition that many, if not most,animals are metaorganisms, comprising host and associated microbial partners, drives a conceptual shift in our view of the hierarchy of life, one that reflects the nested nature of biological systems. Illustrated here is the hierarchy of the fully mature individual and its associated ecological context. Prior to the dawn of next-generation sequencing (‘pre-’), the increasing complexity of the hierarchical organization of biological systems was presumed to be linear, with ‘emergent properties’ arising with each successive level of organization. New data, resulting from advances in sequencing technology (‘post-’), have revealed that the biosphere comprises a more complex hierarchy, one that incorporates the inherent nesting of the macro- and microbial worlds. A holobiont is created through dynamic interactions between the host and microbial elements, through the trajectory of development, from fertilization through ontogeny.