



# Beyond Lynn Margulis' green hydra

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## Abstract

Lynn Margulis has made it clear that in nature partnerships are the predominant form of life; that life processes can only be understood in terms of the interactions of such partnerships; and that their inherent complexity can only be understood by taking a holistic approach. Here I attempt to relate Lynn Margulis' observations on the freshwater polyp hydra to the perceptions and problems of today's Hydra research. To accomplish this, I will synthesize our current understanding of how symbionts influence the phenotype and fitness of hydra. Based on this new findings, a fundamental paradigm shift and a new era is emerging in the way that we consider organisms such as hydra as multi-organismic metaorganisms, just as Lynn Margulis may have thought about it.

**Keywords** Symbiosis · microbiota · Host-microbiome · hydra · Metaorganism · Holobiont · Innate immunity

## 1 Intro Margulis and the discovery of the holobiont

At a time when science was dominated by a mono-causal understanding of life processes, without looking at organisms as a whole beyond their metabolic pathways, Lynn Margulis' scientific contributions were nothing but a true-to-life call for “anti-disciplinary” thinking”. She was not concerned with an absence of discipline, but with the courage to leave the boundaries of the old disciplines and “think around the corner”. A good example of this is her invention of the term “holobiont” (Margulis 1991). She considers organisms as “whole organisms” in the sense of biological systems consisting of a eukaryotic host organism and numerous other eu- and prokaryotic species living closely with it. The term includes the interaction, not just the being together of different organisms with each other. Interaction and cooperation, this is the fundamental rule – and also a “spark of hope” - that Margulis discovered as the driving force in all living beings. As a consequence of this, the “principle of the individual” has been thoroughly overturned by her thinking. This was then eventually reinforced by the consideration (Rosenberg et al. 2010; Rosenberg and

Zilber-Rosenberg 2016; Hunt 2015; Rosenberg 2021) that a focus on an animal genome alone is misleading when it comes to characterize a given biological entity and how it has evolved. Rather than regarding organisms as inherent singularities, a thought provoking and provocative description embraces them as “*vast collaborative enterprises of co-linked, cooperative, co-dependent and competitive ecologies merged together so seamless that they are best considered to be one discrete entity*” (Hunt, 2015). In the meantime, the metaorganism framework has proven to be a powerful new concept to help provide answers to the origin and function of the complex conglomerate of the host and its associated microorganisms (Bosch and McFall-Ngai 2011, 2021; Bosch and Miller 2016; Jaspers et al. 2019; McFall-Ngai and Bosch 2021; <https://www.metaorganism-research.com/de/>).

When Margulis characterized animals as holobionts, she thought above all that they live in symbiosis with other eukaryotes. Just as the freshwater polyp lives with a number of *Chlorella* algae. What is really remarkable is that already at that time Margulis recognized that bacteria could also play an important role in host (hydra) metabolism (Thorington and Margulis 1981). The complexity and function of a microbiome was, of course, still unknown to her at that time. Only with the advent of high-throughput technologies and genomic tools for studying the molecular nature of organisms we learned that in addition to macroscopically visible symbionts, virtually all epithelia are colonized by microbes.

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There is now a multitude of studies indicating that microbes are essential and that a host-specific microbiome provides functions related to the metabolism, immunity, and environmental adaptation of their animal, plant, and fungal hosts (McFall-Ngai et al. 2013; Bordenstein and Theis 2015; Jaspers et al. 2019; Bosch and McFall-Ngai 2021). These multi-species consortia of “microbes” can include bacteria, archaea, protists, fungi, and viruses.

All associated species of the microbiota, i.e. the microbial life forms within a given habitat or host, are considered, regardless of being transient or permanent or whether they form a functional association with the host or other microbes. Species of the microbiome can also exist as free-living organisms, each of which is taken up individually from the environment. Often, however, they are specialized symbionts that cannot exist apart from their host, and are no longer viable on their own. This close living community can, in the course of symbiogenesis, progress to the integration of the symbiont into its host organism. These findings complement and extend Margulis’ view and make it quite clear that symbiotic interactions are not the exception but the rule according to which the functioning of living beings is organized.

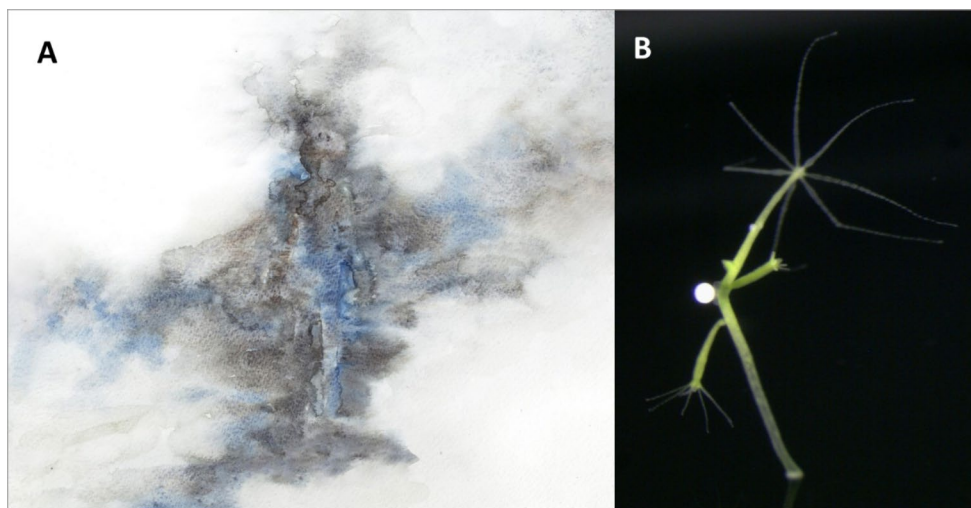
The observations that symbiotic partnerships can achieve all sorts of degrees of intimacy and proximity, in which boundary lines between partners then literally disappear, are not only exciting for scientists. Artists have also taken up these fascinating new ideas and considered how to depict them. An example of how contemporary artists portray the assemblages of an holobiont in which it is not always clear where one member ends and another begins, is an aquarelle by Lukyanova (2020) (Fig. 1 A).

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## 2 Lynn Margulis occupation with hydra. “They have remained in the game of life because they become individuals by incorporation”(Margulis, 1998)

In keeping with the style of her time, Margulis and her co-workers conducted experiments with radioactive substances to decipher the metabolic pathways in a symbiotic partnership and choose the green *Hydra viridissima* (Fig. 1B) as an experimental system (Thorington and Margulis 1981). Hydres were fed with labelled brine shrimp and the fate of the label monitored in animal and algal fractions by scintillation counting. To locate the label, hydra cells and isolated algae were studied by autoradiography using tritiated precursors of protein, DNA, and RNA. They uncovered what they called “back transfer” of metabolites from food to endosymbiotic algae in the digestive cells of *Hydra viridissima*. Specifically they observed that a decrease over time in label introduced as  $^3\text{H}$ -orotic acid and  $^3\text{H}$ -uridine into hydra RNA is compensated for by an increase in label in the algae, implying competition for constant quantities of metabolites from the single feeding. Overall, they discovered that this hydra-*Chlorella* partnership is apparently characterized by a high degree of nutrient transfer between the partners. Despite limited analytical methods, these experiments also made them realize that in addition to algae photobionts there are colonizing bacteria in the *Hydra viridissima* tissue (Margulis et al. 1978; Thorington and Margulis 1981). However, the exact species of bacteria involved and whether, and if so how, the bacteria contribute to the community metabolism remained elusive. Nevertheless, this already very thorough look into a symbiotic community had been a remarkable achievement. It should also be mentioned here that hydra not only fascinated Lynn Margulis’ team, but in the 1980’ s also led numerous other scientists to look into the cellular and metabolic aspects of

**Fig. 1** Holobionts. (A) Wet-on-wet watercolour drawing “Fuzzy relations: depicting holobiont” by Olga Lukyanova. (B) The green holobiont *Hydra viridissima*



the polyp-algae symbiosis. Pioneering studies showed that there is a great deal of adaptation and specificity in this symbiotic relationship. The proliferation of symbiont and host was found to be tightly correlated (Bossert and Dunn 1986; McAuley 1986) with symbiotic *Chlorella* unable to grow outside its polyp host and being transmitted vertically to the next generation of hydra, indicating loss of autonomy during establishment of its symbiotic relationship with this host (Muscatine and McAuley, 1982; McAuley and Smith 1982; Rahat and Reich 1984; Campbell 1990; Habetha et al., 2003). And then the wealth of publications on symbiosis in hydra suddenly ended. Probably because there were no technical possibilities at the time to go beyond what was observed to a deeper functional understanding. As we will see below, thanks to new emerging technologies, in the meantime these early observations could be validated and supplemented.

### 3 The molecularization of hydra and the birth of a model for metaorganism research

Since Trembley's early studies (1744), hydra has been used increasingly as a model system for exploring the principles of development and regeneration. Hydra can be easily maintained in the laboratory as mass cultures. The animals are kept in glass or plastic dishes at 18 °C and fed with brine shrimp nauplii three to four times per week. The simple tube-like body structure is formed by a single layer of ectodermal epithelial cells covered by a multi-layered extracellular cuticle ("glycocalyx"). A single layer of endodermal epithelial cells separates the body from the content of the gastric cavity. The outer layer of hydra's glycocalyx consists of several proteins and glycosaminoglycans and has mucus-like properties (Schröder and Bosch 2016). Hydra are remarkable because they are immortal (Martínez 1998; Boehm et al. 2013; Nebel and Bosch 2012) and can live an estimated 1,400 years (Jones et al. 2014). Much of immortality can be traced back to the asexual mode of reproduction by budding which requires a tissue consisting of stem cells with continuous self-renewal capacity. Stem cell differentiation in Hydra is governed through the coordinated actions of conserved signalling pathways. Emerging novel technologies and the availability of genomic and transcriptomic resources allow to analyse these developmental processes in vivo. The *Hydra magnipapillata* (cf. *Hydra vulgaris*) genome was made available in 2010 (Chapman et al. 2010), and this was soon followed by the establishment of a reference transcriptome (Wenger and Galliot 2013), quantitative RNA-sequencing (Hemrich et al. 2012; Vogt et al. 2019; Rausch et al. 2019), quantitative proteomics, and single cell sequencing (Siebert et al. 2019; Klimovich et al. 2020).

Molecular tools exist to analyse gene function in adult and regenerating animals. Stable transgenesis was established in 2006 by using an embryo microinjection technique (Wittlieb et al. 2006; Klimovich et al. 2019). The diversity of available construct designs ensures a broad application range of the transgenic hydra technology and allows constitutive gene gain- and loss-of-function analyses, as well as conditional gene manipulation, dissection of cis-regulatory sequences, differential labeling and in vivo visualization of the entire repertoire of cell types in a polyp. In addition, the visualization of hydra development and regeneration has advanced in recent years, with the addition of fluorescent reporters and sophisticated live-imaging approaches (Aufschnaiter et al. 2011; Carter et al. 2016; Szymanski and Yuste 2019; Giez et al. 2021).

Finally, a new generation of sequencing technologies has uncovered that hydra is colonized with microbes. Hydra's ectodermal epithelial surface is densely colonized by a stable multispecies bacterial community (Fraune and Bosch 2007; Franzenburg et al. 2013). The symbiotic partnership is driven by interactions among the microbiota and the host. The outer glycocalyx layer provides the habitat for a specific bacterial community (Fraune et al. 2015; Schröder and Bosch 2016). This extracellular pattern - an inner layer with stratified organization devoid of bacteria, beneath an outer loose layer colonized by symbionts -- is similar to the mammalian colon (McFall-Ngai and Bosch 2021). The asexual mode of reproduction by budding allows to maintain the hydra microbiota by vertical transmission. The colonization process depends on host-derived factors as well as interactions between individual bacteria (Franzenburg et al. 2013).

The simple architecture of the hydra body, the limited number of different bacterial symbionts, and the experimental accessibility of both host tissue and colonizing microbes have made hydra an excellent model organism for studying host-microbe interactions (Klimovich and Bosch 2018; McFall-Ngai and Bosch, 2021; Lousada et al. 2022). The microbiome provides colonization resistance and thereby prevents pathobiont invasion (Bosch 2013; Fraune et al. 2015; Rathje et al. 2020). The colonizing microbes also stabilize the patterning mechanisms by acting on the biochemical processes controlling the continuous self-renewal and differentiation capacity of stem cells (Taubenheim et al. 2020; He and Bosch, unpubl.). The hydra host shapes the specific microbiome by means of the innate immune system and a rich repertoire of antimicrobial peptides (Franzenburg et al. 2013; Bosch 2014). 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 and Bosch 2007). Ecto- and endodermal epithelial cells as well as neurons produce a rich repertoire of antimicrobial

peptides that in hydra (Franzenburg et al. 2013; Augustin et al. 2017; Klimovich et al. 2020) as in many other animal taxa (Bosch and Zasloff 2021) shape the microbiome.

The microbiota follows a distinct spatial colonization pattern along the body column (Augustin et al. 2017) with some symbionts predominantly colonizing the oral region and others the aboral region of the body column. Microbial colonizers in hydra can be tracked via fluorescent dye and used to understand in vivo microbial colonization dynamics. Recolonization experiments of both non-sterile and germfree animals using stable (chromosomal) fluorescent labelled *Curvibacter* bacteria showed that the spatial pattern is only visible in non-sterile polyps but not in germ-free polyps (with all niches un-occupied) (Wein et al. 2018). These observations hint to interesting mechanisms how spatial pattern might be maintained and established. Hydra's stable associated microbiome is amenable to extensive manipulations (Wein et al. 2018; Murillo-Rincon et al. 2017). Microbial colonizers in hydra can be tracked via fluorescent dye and used to understand in vivo microbial colonization dynamics and therefore should enable deep insight into the fundamental principles of control of microbial biogeography. Experimental approaches include microbial transplantation experiments of germ-free polyps using either single bacterial strains or different combinations of hydra specific bacterial isolates (Murillo et al. 2017). Moreover, metabolites produced and secreted by bacteria after transplantation when living in the mucosal surface environment of hydra can be analysed by conducting metabolomics of the surrounding water.

#### 4 A late homage to Margulis: genomic approaches confirm metabolic dependency as base for the ancestral Hydra-*Chlorella* symbiosis

Recent advances in transcriptome and genome analysis allowed us to identify the metabolic interactions and genomic evolution involved in achieving the hydra-*Chlorella* symbiotic relationship (Hamada et al. 2018). In an unbiased approach we identified key players that control the symbiosis between *Hydra viridissima* and its photosynthetic symbiont *Chlorella* sp. A99. To achieve this, we have sequenced the entire genome of *Chlorella*. This revealed that *Chlorella* has lost some of the genes required to obtain nitrates, and to process them into nitrogen. However, the genetic analysis showed that the algae express genes that allow them to import amino acids. In turn, analysis of the genes expressed by hydra when it lives in symbiosis with *Chlorella* showed that the animal turns on genetic information needed to make glutamine. It thus seems that hydra creates glutamine which

*Chlorella* can import; the algae then process this amino acid to obtain the nitrogen they need. Another related observation of potentially practical importance was that we could finally maintain *Chlorella* in *in vitro* culture when we artificially enriched the medium with glutamine. For the first time ever, the photobiont *Chlorella* could live on their own outside of hydra, at least for a while (Hamada et al. 2018).

The results suggest that symbiotic relationships, such as the one between hydra and *Chlorella*, were established because the organisms became dependent on each other for essential nutrients. This co-dependency is strengthened if the organisms lose the ability to produce the nutrients on their own. As already observed and predicted, at least in part, by Margulis, the exchange of nutrients is the primary driving force for the symbiosis between *Chlorella* and hydra.

#### 5 In a tripartite relationship, everything is once more complicated: in the hydra host, *Chlorella* interacts with the microbes

Symbiosis research traditionally focused on bipartite host-microbe interactions such as the plant root-Rhizobium symbiosis or algae-fungi symbiosis in lichen, while tripartite and multipartite associations received lesser attention. However, it is becoming evident that long-term symbiotic persistence is prevalent not only as two-party but also as more complex multipartite systems. At the bottom of the Thorington and Margulis paper (1981), there is the very telling sentence: “Our results and those of Wilkerson (1980) suggest that further quantitative studies of metabolic interactions in hydra symbioses must consider the roles of all three partners in nutrient flow: *Hydra viridis* (host) (now termed *Hydra viridissima*), *Chlorella* sp. (symbiotic algae), and *Aeromonas punctata* (symbiotic bacteria) cells”. This was written in the 80s. Today, 40 years later, it becomes obvious that in the ménage à trois in hydra, the symbiotic bacteria indeed are participating in addition to the polyp and the algae. First experimental insights show very clearly not only how complex interactions in the metaorganism hydra are in reality, but also demonstrate the beneficial role of the endosymbiotic *Chlorella* photobiont in maintaining a species-specific microbiome (Bathia et al. 2022). When one removes the algae and cultures aposymbiotic (algae free) *H. viridissima* polyps, their microbiome is significantly different and dominated by bacteria of the *Legionella* group. *Legionella* is a common habitant in hydra's freshwater habitat and in the laboratory culture apparently acquired from the culture medium. Culturing aposymbiotic and symbiotic polyps in a shared environment allows horizontal transmission of bacteria from aposymbiotic animals to symbiotic polyps and a dramatic alteration of the microbiome in symbiotic

animals. Transfer of *Legionella* from aposymbiotic to symbiotic polyps causes the algae to temporarily leave the hydra epithelium and also to a decreased growth rate of polyps, indicating considerable fitness costs for symbiotic animals. How the *Chlorella* algae leave the hydra tissue is not known. Interestingly, over a longer period of time, recolonization of aposymbiotic animals with *Chlorella* algae results in the restoration of the microbiome to the native state of monocultivated symbiotic animals. The molecular mechanisms controlling these interactions are still unknown to us. What we can state for sure is that the tripartite interactions are complex. And that the *Chlorella* photobiont is involved in controlling invading bacteria and stabilizing the resident microbiota (Bathia et al. 2022). This is of relevance because inter-host dispersal of bacteria is not a rare phenomenon. Transmission of microbes from one individual to another one in a shared environment has been documented previously for aquatic systems, as exemplified in corals (Grupstra et al. 2021) and zebrafish (Burns et al. 2017). Moreover, co-housing of mice has a profound effect on their gut microbiome (Caruso et al. 2019). It needs reductionistic model systems such as hydra for a functional understanding of the mechanisms involved.

## 6 40 years after Margulis, the hydra reimaged

The association between microorganisms and animals has important evolutionary consequences at scales ranging from the health and fitness of the participating organisms to the rates and patterns of evolutionary diversification of both animals and their microbial partners. In both vertebrates and invertebrates a complex microbiome confers immunological, metabolic and behavioural properties; its disturbance can contribute to the development of disease states. The evolutionary dynamics within such a metaorganism and the involved molecular interactions are complex and the molecular and cellular mechanisms controlling the interactions are still poorly understood.

Here I considered how an appreciation of the microbiology of the freshwater polyp hydra is changing our understanding of the origin and functioning of animals. Uncovering the symbiotic interactions in hydra 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 et al

2015). Moreover, microbial metabolites not only stimulate hydra's immune response but also influence spontaneous body contractions (Murillo-Rincon et al. 2017) and reactive behaviour such as the feeding reflex (Giez and Bosch, unpubl.). And as if that is not enough, recent observations on long-term germ-free animals indicate, that hydra's developmental pathways and specifically the stem cell systems are dependent on the presence of the microbiota (He and Bosch, unpubl.).

Our observations in hydra allow two important general conclusions. First, there is a need to consider the multi-organismic and holobiotic nature of an organism surrounded by its microbial counterparts when thinking about the "functioning" of an animal. And second, the microbial environment and the presence of symbionts matter and contribute to complex processes such as development (Carrier and Bosch 2022). Offering a new and evolutionary informed perspective, we argue here that decoding the mechanisms of how hydra communicates with its microbiome can provide a general understanding of the tightly linked interactions between host environment, nutrient dependency of host-associated microbes, microbial metabolism, microbe-microbe interactions and host immunity.

Some future priorities for hydra symbiosis research are to experimentally assess and validate general molecular and evolutionary principles shaping the metaorganism and to contribute to a novel integrated understanding of complex symbiotic communities and their association with hosts. We are particularly interested in the specific functional consequences of the interactions, the underlying regulatory principles, and also the resulting impact on host life history and evolutionary fitness in the selected host systems.

No one has highlighted hydra's singularity in today's certainly competitive symbiosis research better than Lynn Margulis. I therefore conclude this particular chapter by quoting the last sentence from her paper (Thorington and Margulis 1981): *The separability, manipulability, and experimentally achieved independent growth of the three partners in this symbiosis, coupled with the diverse quality and large quantity of metabolite flow, makes Hydra viridis (now termed Hydra viridissima) and its microbes ideal for testing models of the evolutionary origin of symbioses*". Lynn Margulis could not know that for sure, of course. It takes a strong will to turn an idea into a success. And, of course, the people who support it and help make it happen.

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