Competing forces maintain the *Hydra* metaorganism

Peter Deines*, Tim Lachnit* and Thomas C.G. Bosch

Zoological Institute, Christian Albrechts University Kiel, 24098 Kiel, Germany

Postal address:
Zoological Institute
Christian Albrechts University Kiel
Am Botanischen Garten 1-9, 24118 Kiel, Germany
Tel: +49-431-880-4169
Fax: +49-431-880-4747
Email address of corresponding author: tbosch@zoologie.uni-kiel.de

* These authors contributed equally to this work.

RUNNING TITLE

Maintenance of homeostasis in a metaorganism

SUMMARY

Our conventional view of multicellular organisms often overlooks the fact that they are metaorganisms. They consist of a host, which is comprised of both a community of self-replicating cells that can compete as well as cooperate and a community of associated microorganisms. This newly discovered complexity raises a profound challenge: How to maintain such a multicellular association that includes independently replicating units and even different genotypes? Here we identify competing forces acting at the host tissue level, the host-microbe interface, and within the microbial community as key factors to maintain the metaorganism *Hydra*. Maintenance of host tissue integrity, as well as proper regulation and management of the multiorganismic interactions are fundamental to organismal survival and health. Findings derived from the *in vivo* context of the *Hydra* model may provide one of the simplest possible systems to address questions of how a metaorganism is established and remains in balance over time.

KEYWORDS

metaorganism, symbiosis, innate immunity, multiorganismic interactions, microbiota, homeostasis

1. Introduction

The «metaorganism» concept (1-4) considers the dynamic communities of microorganisms on epithelial surfaces as an integral part of the functionality of the respective organism itself. Today there is also an increasing appreciation that microbes are an essential part of the animal phenotype influencing fitness and thus ecologically-important traits of their hosts (5-7). Disease onset is seen as a complex set of interactions among a variety of associated partners that affect the fitness of the collective metaorganism (8). Discovering that individuals are not solitary, homogenous entities but consist of complex communities of many species that likely evolved during a billion years of coexistence led to the hologenome theory of evolution (1, 9, 10) which considers the holobiont with its hologenome as the unit of selection in evolution.

Box 1: Terminology Metaorganism

Holobiont: Is an eukaryotic host with all its associated microbial partners. This multispecies assemblage includes viruses, phages, eubacteria, archaea, fungi and protozoa.

Hologenome: Genetic information encoded in the eukaryotic host and all of its associated partners. This collective genome forms the theoretical genetic repertoire of a holobiont.

Metaorganism: Includes the function of a holobiont in a given environment. The function of a holobiont depends on I) presence and composition of the associated partners, framing the genetic potential of the holobiont the hologenome; II) the activity, abundance and the transcriptional active part of the genome of every single partner of the holobiont; III) this subsequently results in interactions between host-

78 microbes and microbe-microbe which finally must be retained at homeostasis in
79 order to maintain a stable holobiont. To emphasize this highly dynamic functional
80 state (capacity) of a holobiont we refer to in the following as metaorganism.
81
82
83 Current research is focused on understanding the general principles by which these
84 complex host-microbe communities function and evolve. Which selective forces drive
85 the evolution of these interactions, i.e. how do the associated organisms influence
86 each other's fitness? Which forces shape the colonizing microbial composition? The
87 recognition that microbes are an integral part of higher organisms, and that they live
88 in a complex and stable community with dynamic interactions both internally and
89 towards the host, often results in the misunderstanding of considering these
90 interactions as purely beneficial and cooperative. In reality, interactions within a given
91 holobiont can range from cooperative to competitive to even parasitic. While in
92 cooperative interactions both partners benefit from each other, competition usually
93 results in resource partitioning.
94
95 Due to progress in deep sequencing in the last decade, we got accustomed to the
96 idea of organisms as holobionts and the complexity of interactions between host and
97 associated microbial cells (metaorganisms). However, we often forget that in addition
98 to interactions between host cells and microbes, multicellular organisms per se are a
99 complex “society of cells” (11, 12) consisting of independently replicating cells which
100 adapt their replication rate to the environmental condition. These considerations
101 indicate that ensuring functional homogeneity of tissue and maintaining a
102 multicellular collective should be considered a multi-level phenomena that extend
103 from the cell- to the tissue- to the organismal – and ultimately to the meta-organismal

levels. The considerations also raise a profound and largely unexplored challenge: what are the mechanisms allowing an organism to function as a multicellular association of independently replicating cells of different genotypes? From an evolutionary biology perspective, multicellular organisms are the result of a “major evolutionary transition“ in individuality, where previously independently replicating cells gave up their right on autonomous replication to reproduce only as part of the higher level entity (11, 13-15). Resolution of conflict between the cells appears key to such a transition.

Here we introduce *Hydra* as a valuable model for exploring the competing forces in a metaorganism. *Hydra* is member of the animal phylum cnidaria which are not only among the earliest known phyletic lineages known to contain stem cells as well as neurons but also possess most of the gene families found in bilaterians (16-20). Similar to other animals, cnidaria are multicellular complex holobionts consisting of the diploblastic animal host and its associated endogenous microbiota. In *Hydra*, host tissue integrity and multicellular organization are defended by both an elaborate innate immune response (21) and phagocytic processes (22, 23) which together form a robust and critical system through which self is distinguished from non-self, pathogenic signals are recognized and eliminated, and host tissue homeostasis is maintained. In addition, inter-species interactions between the host and its stable microbiome, interactions between photosynthetic algae and their host cells, as well as interactions within the microbial community (24) are further important components of the *Hydra* metaorganism. Disturbance or shifts in any of these interactions partners can compromise the health of the whole animal (25). Since the uncovered basic molecular machinery can be transliterated to more complex organisms and promises to provide conceptual insights into the complexity of host-microbe interactions, an in-depth knowledge of the basic biology of each of the members of

the *Hydra* holobiont and the corresponding interactions might be informative to understanding more complex metaorganisms such as vertebrates and humans. This comparison seems to be important in light of the increasing number of chronic and non-communicable diseases observed in the last decades and the need for testing the hypothesis that microbial and other environmental challenges are the main causative factors of disease manifestation in genetically susceptible individuals.

2. Cell-cell competition in the animal host

Hydra is a unique model system to study tissue homeostasis due to its extraordinary regenerative capacity and the continuous self-renewal and differentiating potential of its epithelial and interstitial stem cells. These properties are related to the fact that these animals continuously reproduce asexually by budding (26-28). Regeneration and continuous self-renewal is due to the presence of three stem cell lineages: ectodermal and endodermal epithelial cells and interstitial stem cells (29). The longterm persistence of three independent stem cell lineages in a given organism represents a profound challenge to the animal: how to maintain a cellular collective comprised of reproductively independent cells in a constantly changing environment? From the molecular view, autophagy and apoptosis are generally seen as key mechanisms that maintain the whole organism at the expense of individual cells (30-32). Autophagy is a cell protective process with a role in nutrient starvation (33). When nutrients are restricted, cells elaborate double-walled membranes known as phagophores, which enclose cell constituents to form autophagosomes that subsequently fuse with lysosomes to produce autophagolysosomes. Studies of nutrient deprivation in *Hydra* have shown that well-fed animals starved for 10 days

start to induce autophagy (34). In addition, epithelial cells in *Hydra* also possess an intrinsic defence mechanism against competing neighbours which is strictly environment dependent and was described previously (22) as apoptosis. *Hydra* polyps grow continuously due to proliferation of epithelial and interstitial stem cells throughout the body column. However, polyps do not increase in size since cells are continuously transferred to asexual buds, which form on the lower body column, and are lost at the tentacle tips and in the basal disk. Budding is dependent on feeding: well-fed polyps produce roughly one bud per day; starved polyps cease to form buds after 1–2 days. Unexpectedly, our early work has shown that this striking dependence of budding on feeding is not due to a change in cell proliferation, as initially anticipated, but rather to apoptosis (22). Rapid cell proliferation detected as an increase in the 3H-thymidine labeling index occurs in both well-fed and starved animals. The increase in cell numbers, however, is dramatically different: cell numbers increase exponentially in fed animals but do not change in starved animals. This difference is due to an increased rate of apoptosis in starving polyps. Bosch and David (22) observed a 7-fold increase in epithelial cells containing phagocytized apoptotic bodies in starving polyps compared to well-fed polyps. While these observations clearly indicate that environment-dependent elimination of cells from the epithelium - which we consider to be some form of cell competition - regulates growth in *Hydra*, the important question remains as to which molecular regulators are involved in inter- and intracellular clearance? Studies have consistently revealed that FoxO (Forkhead box O) transcription factors play an important role in stem cell biology and tissue homeostasis. During aging, for example, the balance of removal and regeneration of cells in tissues becomes disturbed mainly due to a decrease in the regenerative potential of adult stem cells. Conditional deletion of FoxO1/3a/4 in the adult hematopoietic stem cell system of mice leads to apoptosis of hematopoietic

stem cells preventing the repopulation of these stem cell populations. Similarly, aged mice in which FoxO3a was deleted display reduced regeneration potential (35, reviewed in 36).

To uncover the molecules controlling the continuous self-renewal and differentiation in *Hydra* we used a transcriptomic approach to identify the molecular signatures of *Hydra´s* three stem cell lineages. We showed that FoxO is highly expressed in all three stem cell lineages (37, 38). Overexpression of FoxO in the multipotent interstitial stem cell lineage increased stem and progenitor cell proliferation and activated expression of stem cell genes such as nanos in terminally differentiated somatic cells such as nematocytes (37). Conversely, silencing FoxO in epithelia cells increased the number of terminally differentiated cells and slowed down growth rate (37). Previous work has discovered significant parallels in the regulation of FoxO between *Hydra* and bilaterian animals (39, 40). Together with our functional studies in transgenic *Hydra*, these results suggest a key role for FoxO in *Hydra´s* remarkable ability to continuously maintain tissue homeostasis. The environment dependent control of tissue homeostasis raises the question, whether FoxO activity is directly involved in the interaction with the environment.

3. Competing forces between the *Hydra* epithelium and the colonizing microbes: key roles of AMPs

For decades a number of *Hydra* species have been cultivated under standard conditions at constant temperature and identical food. It came as a complete surprise, therefore, that examining the microbiota in different *Hydra* species kept in

208 the laboratory for more than 20 years under controlled conditions revealed an
209 epithelium colonized by a complex community of microbes, and that individuals from
210 different species differed greatly in their microbiota. Even more astonishing was the
211 finding that individuals living in the wild were colonized by a group of microbes that is
212 similar to that in polyps grown in the lab, pointing to the maintenance of specific
213 microbial communities over long periods of time. Bacteria in *Hydra* are specific for
214 any given species (41, 42). Closely related *Hydra* species as *Hydra vulgaris* and
215 *Hydra magnipapillata* are associated with a very similar microbial community. In
216 contrast, *Hydra oligactis,* the most basal *Hydra* species analysed so far (43), is
217 associated with the most distinct microbial community compared to the other *Hydra*
218 species. In line with this, comparing the phylogenetic tree of the *Hydra* species with
219 the according cluster tree of associated bacterial communities reveals a high degree
220 of congruency (42). This strongly indicates that distinct competing forces are
221 imposed on and within the *Hydra* epithelium.
222
223 In the absence of an adaptive immune system, *Hydra* employs an elaborate innate
224 immune system to detect and interact with microbes using their two cell layers as
225 efficient defense barriers (44). Invading microorganisms first have to overcome the
226 physicochemical barrier represented by the multilayered glycocalyx that covers the
227 ectodermal epithelium (45). Complex cellular and humoral pathways represent the
228 second arm of *Hydra's* immunity (21). Cellular mechanisms include phagocytosis,
229 tissue repair and regeneration, and apoptotic reactions. Apart from these cellular
230 mechanisms, *Hydra* possesses a broad range of antimicrobial factors such as
231 antimicrobial peptides (AMPs; *Fig. 1*) and kazal 2-type protease inhibitors (44).
232

Antimicrobial peptides (AMPs) produced in adult polyps include hydramacin (21) and arminin (46) to control bacterial colonization via MyD88 (47; *Fig. 2*). Our previous work has shown that AMPs have in addition to their killing activity against pathogens clear regulatory functions in host-microbe homeostasis and are considered as the driving force that leads to changes in microbiota composition. To investigate whether the ectotopic expression of an AMP may affect the number and composition of the colonizing microbiota at the ectodermal epithelial surface, we generated transgenic *Hydra* expressing periculin1a in ectoderm epithelial cells (48). Comparing the bacterial load of these transgenic polyps with that of wild-type control polyps revealed not only a significantly lower bacterial load in transgenic polyps overexpressing periculin1a but also, unexpectedly, drastic changes in the bacterial community structure. Analyzing the identity of the colonizing bacteria showed that the dominant β-Proteobacteria decreased in number, whereas α-Proteobacteria were more prevalent. Thus, overexpression of periculin causes not only a decrease in the number of associated bacteria but also a changed bacterial composition. With the transgenic polyps overexpressing periculin we apparently have created a new holobiont that is different from all investigated *Hydra* species. From these results we assume that specific associations between hosts and bacteria are a result of bacterial adaptation to different repertoires on AMPs in different host species. Evolutionary changes in the AMP repertoire of host species, therefore, are expected to lead to changes in the composition of the associated bacterial community. These findings support the view that epithelial-derived AMPs are an important regulatory force shaping the composition of epithelial microbiota (*Fig. 2*).

Interestingly, and of significance in the context of environment-dependent control of tissue homeostasis, AMPs were recently discovered to be direct target genes of

transcription factor FoxO. Besides its well-known conserved function as major tissue regulator, FoxO modulates the innate immune system in various model organisms including *Drosophila* (49, 50), *C. elegans* (51) and *Hydra* (37). In *Hydra*, the microbiome is selectively assembled by a species-specific combination of AMPs which are predominantly expressed in epithelial cells (42). Remarkably, loss of tissue homeostasis as well as AMP-deficiency result in a decreased potential to select for microbial communities resembling the polyps native microbiota (25, 42). Transgenic *Hydra* polyps in which the single FoxO gene is down-regulated show in addition to problems in stem cell maintenance a severe change of the immune status and drastically altered expression of AMPs (37). AMPs are also in *Drosophila* well known effector molecules of the innate immune system and important regulators of the bacterial colonizers. Here, oral microbial infection induces FoxO activity in the intestine, while impaired FoxO signaling decreases resistance to intestinal infections. The inability to raise the expression level of AMPs leads to an elevated bacterial load and a decline in survival (52). Thus, transcription factor FoxO appears to combine two functions crucially involved in tissue homeostasis and health in metazoans: FoxO is responsible for stem cell regulation, including tissue maintenance and renewal, and controls the innate immune system. In response to environmental (or bacterial) signals FoxO shuttles between an transcriptionally inactive state in the cytoplasm and an active form in the nucleus thereby serving as an intracellular control board for environmental signals.

The intimacy of the interaction between host and microbiota, as well as the high evolutionary pressure to maintain a specific microbiota, points to the significance of the interkingdom association and implies that hosts deprived of their microbiota should be at a disadvantage. To investigate the effect of absence of microbiota in

285 *Hydra* we have produced gnotobiotic *Hydra* polyps that are devoid of any bacteria.
286 While morphologically no differences could be observed to control polyps, we
287 presented evidence that *Hydra* lacking bacteria suffer from fungal infections unknown
288 in normally cultured polyps (53). Removing the epithelial microbiota results in lethal
289 infection by the filamentous fungus *Fusarium sp.*. Restoring the complex microbiota
290 in gnotobiotic polyps prevents pathogen infection. While mono-associations with
291 distinct members of the microbiota fail to provide full protection, additive and
292 synergistic interactions of commensal bacteria are contributing to full fungal
293 resistance. These observations highlight the importance of resident microbiota
294 diversity as a protective factor against pathogen infections.
295
296 Observations in a number of other invertebrates and vertebrates strongly support the
297 view that in addition to being integral components of the innate immune system,
298 microbes should also be considered partners in animal development. Bacterial
299 contributions are indispensable, for example, in shaping the immune system and
300 development of organs such as the vertebrate intestine or the squid light organ
301 (reviewed in 7). Animal development has traditionally been viewed as an
302 autonomous process directed by the genome. It seems that we have to rethink
303 development at least in part, as an orchestration of both animal-encoded ontogeny
304 and inter-kingdom communication. The beneficial microbiota is a complex and
305 multifunction ecosystem that is essential to the development, protection, and overall
306 health of its host. Thus, the microbiota appears to function as an extra organ, to
307 which the host has outsourced numerous crucial metabolic, nutritional, and protective
308 functions. Studies from cnidaria to primates indicate that the host's role far outweighs
309 other environmental factors in molding the composition of the microbiota. AMPs
310 appear to be key factors for host-bacteria co-evolution and the driving force that

leads to changes in microbiota composition. Finally, and maybe most important, the dynamic relationship between symbiotic microorganisms and environmental conditions results in the selection of the most advantageous holobiont.

4. Competing forces are also the key components in shaping the bacterial community

Microbial species rarely exist in isolation or as single species populations but rather as dense and often diverse communities as detected by several studies in a range of habitats (54, 55). This suggests that microbial interactions play a pivotal role in the establishment and resilience of populations in different abiotic environments. The same is thought to be true for eukaryotic organisms as they function as environments for their associated microbes and have been co-evolving with them. That is evident in host mechanisms that do not simply exclude all microbes from the environmentally exposed host surfaces but finely regulate the associated bacterial communities (56). This can also be observed for the host *Hydra*, where the associated microbiome is not a random assemblage of bacteria from the environment, but a very specific community despite the fact that the polyps are in continuous close contact with the surrounding bacterioplankton (41, 42, 44). From the available pool, bacteria are selectively recruited, depending on host immunity and genetic background (42, 44), but also on the interactions between the co-occurring microbes, host physiology, and the specific environmental conditions (57, 58). Evidence has accumulated that hosts should be viewed as “ecosystem engineers that manipulate general, system-wide properties of microbial communities to their benefit” (59).

337

338 ***Microbial colonization of Hydra***

339

340 Before colonization, microbes must reach a host's surface, likely through diffusive or
341 convective passage and active swimming (60). In a recent article Tout and
342 colleagues (61) suggest that motility and chemotaxis are important bacterial traits for
343 the establishment of specific coral-bacterial interactions. They outline the mechanism
344 through which chemical gradients associated with coral surfaces attract particular
345 microbial species and so lead to the specific composition of coral reef bacterial
346 communities.
347 This might also be true for *Hydra*, as motility and chemotaxis are prevalent traits
348 among the *Hydra*-associated bacteria (62, Deines, personal communication).
349 Moreover, evidence is accumulating that the colonizing bacteria sense and respond
350 to *Hydra's* chemical landscape and actively move towards the host (Deines, personal
351 communication). It is very likely that the colonization of *Hydra* already occurs on a
352 very fine scale, as a specific microbial composition is associated with distinct parts of
353 its body (Augustin, personal communication). Such a colonization of a preferred
354 surface microenvironment is known from biofilms, where bacteria respond to very
355 distinct environmental signals, enabling them to occupy their specific niche (63).
356 A critical step in the process of colonization is the adhesion to a surface, which can
357 either be reversible or irreversible (64). It is postulated that the colonization potential
358 of a bacterium on various substrates can be described by its "secretome", which
359 includes both the secretion systems and their protein substrates (64). This concept
360 offers not only a lot of potential in terms of investigating colonization factors in the
361 context of infection but also in determining their involvement in the colonization of
362 host species by their specific microbiota (64). It is however unlikely that hosts are

merely passive bystanders in the colonization process as there is selection on hosts for managing their microbiome (65). The role of host factors in regulating microbial adhesion at epithelial surfaces has recently been addressed by McLoughlin et al. (66). Using an individual-based modeling approach, they predict that the host changes the competitive potential of particular microbes and can also create refugia for slow-growing species. The host can for example select for or against certain microbes through the release of specific adhesive molecules from its epithelial surfaces or through an increase in mucus flow respectively. There is evidence from the *Hydra* system that supports the model prediction that the host selects for specific microbes. When studying the population dynamics of the two main colonizers of *Hydra* (*Curvibacter sp.* (AEP1.3) and *Duganella sp.* (C1.2)) *in vitro Duganella sp.* quickly outgrows *Curvibacter sp.* and eventually pushes it towards extinction irrespective of their initial frequencies (67). This is in contrast to the relative abundances found on the host. Here, *Duganella sp.* is only the second most dominant colonizer with 11.1%, and not able to outcompete the main colonizer *Curvibacter sp.* that reaches 75.6% (53). Such frequencies are also reached when letting both bacterial species colonize sterile *Hydra* at different initial frequencies. In contrast to the *in vitro* findings, on the host *Curvibacter sp.* is able to outcompete the faster growing *Duganella sp.* strain. This showcases the role of the host in controlling and shaping the abundance and diversity of its microbiome. Whether this result is due to host-secretions, host-epithelial feeding, host immunity or a combination of all is currently being investigated.

Hydra can reproduce either asexually or sexually. Under favorable conditions *Hydra* reproduces via asexual budding (68). When population densities are high or environmental conditions deteriorate, *Hydra* reproduces sexually through the

formation of ectodermally-located testis and oocytes (69). Following fertilization, oocytes develop outside the female. Embryonic development begins with radial cleavages forming a coeblastula about eight hours post fertilization, subsequently followed by gastrulation (*Fig. 2*). At the end of gastrulation, about 24 hours post fertilization, cells of the outer layer develop filopodia (spike stage), and finally secrete cuticular material forming a thick multilayered protective structure ending in the cuticle stage (three days after fertilization) (70). After a variable period of time (two to 24 weeks) the small polyp hatches from the cuticle with its head first. It has been shown that each of these different developmental stages serve as a substrate for a specific set of microorganisms (48, 71). Early embryos, for example, harbor significantly fewer bacteria than later developmental stages, such as spike and cuticle stage. This result is likely caused by an effective and specific antimicrobial defense system, which has been termed *Hydra's* "be prepared" embryo-protection strategy (48). This early defense is composed of maternally synthesized antimicrobial peptides of the periculin family that shape the initial colonizing bacterial community. The cuticle stage in contrast is characterized by a ~30 fold increase in bacterial load. One explanation for this could be that this is a stage where the host does not possess any control, and it thus functions as a passive settling substrate for the bacteria (48). Alternatively the host could also actively promote growth and attachment of a very specific bacterial community by host-epithelial feeding (spike and cuticle stage are characterized by an additional outer matrix). This could form the starting community for *Hydra* hatchlings eclosing from the cuticle. At present it is unclear whether the environment within the cuticle is germ-free or whether it is also colonized by specific bacteria. These bacteria could be of major importance for the eclosing process of the hatchling or for later development and growth. Recent evidence from humans suggests that such a scenario is not unlikely. Collado and

415 coworkes proposed that the stepwise microbial gut colonization process may be
416 initiated already prenatally/*in utero* by a distinct microbiota in the placenta and
417 amniotic fluid (72). Whether a prenatal bacterial microbiota exists across the tree of
418 life is as yet unknown.

419 After the *Hydra* hatchling successfully eclosed from the cuticle, its epithelium is
420 colonized by microbes from the environment and the outside (and potentially inside)
421 of the cuticle (*Fig. 2*). Colonizing bacteria are most likely attracted through host
422 metabolites, i.e. through the specific chemical landscape of the *Hydra* hatchlings (see
423 above for more detail). Once microbes have reached a suitable niche, for example a
424 host, they must establish themselves through physical attachment to the niche or
425 they will drift away. This can happen via bacterial capsular polysaccharides or
426 appendages such as pili and fimbrae with which bacteria can either directly attach to
427 the host tissue, its extracellular proteins, or other microbes with which they form
428 biofilms (73). Resources for bacterial survival and reproduction either stem from the
429 surrounding environment, the host, or from other neighbouring microbes (for possible
430 metabolic interactions between microbes see Box 2). Essential resources for
431 microbes comprise of micronutrients such as iron and salts and macronutrients such
432 as complex carbohydrates as indicated by a recent study on the mice intestinal
433 microbiome (74).

434 The succession of the microbial colonization of *Hydra* hatchlings was monitored for
435 up to 15 weeks (71), and found to go through defined and reproducible stages (*Fig.*
436 *2*). A high number and rich diversity of bacterial species characterized the initial
437 colonization phase, which was replaced in the second week by a transient adult like
438 profile. Four weeks after hatching a stable adult-like pattern emerged, characterized
439 by a low diversity microbiome that was dominated by the species that are
440 characteristic for *Hydra's* adult stage with the predominance of *Curvibacter sp.*. With

441 the help of a theoretical model, the cause of the observed microbial colonization
442 pattern was predicted to likely be caused by both, host factors, such as the innate
443 immune system, and frequency-dependent bacteria-bacteria interactions (71). The
444 host immune response is thought to reduce the fluctuations in bacterial community
445 dynamics, whereas the composition of a stable microbiome seems to depend upon
446 initial colonization of one (later the most abundant) community member (71).
447 These results are in line with more general predictions, where one or few “keystone
448 species” are founders of the community and determine the ultimate composition and
449 function of e.g. the human gut microbiome. This concept stems from conservation
450 biology but has successfully been transferred to bacterial community composition in
451 a diverse range of ecosystems (75, 76). It is thought that the host in turn controls his
452 microbial community my managing the “keystone species”, rather than controlling
453 each microbial species of its rich microbial community individually. This has been
454 also recently shown for plant microbiomes (77), where particular microbes, termed
455 “hub microbes”, have been found to be disproportionally important in shaping the
456 microbial community in the phylosphere (e.g. controlling the abundance of other
457 bacteria). Importantly microbial “hubs” are strongly interconnected and take a central
458 position in their microbial networks. The identification of “keystone” or “hub” species
459 are promising targets for controlling host-associated microbial communities in health
460 and disease, and may open up new avenues for the identification of bacteria that can
461 specifically been targeted.
462 Another component that might contribute to the predominance of *Curvibacter* in
463 *Hydra* is the virome (see below). Current evidence suggests that the virome is
464 responsible for modulating the structure and function of host associated communities
465 (78; *Fig. 1*).
466

467

468 ***Stability of bacterial communities in Hydra – the central role of competing***
469 ***forces***

470

471 Bacterial communities are species assemblages that occupy a specific habitat where
472 they compete for environmental resources. These complex multispecies communities
473 can be remarkably stable and resilient, examples include microbial mats in the ocean
474 and host associated microbiomes such as the gut of many insects and animals and
475 humans (58, 79, 80, 55). A stable microbiome can also be observed in *Hydra*. Here
476 polyps in their natural environment and individuals that have been maintained under
477 laboratory conditions for >30 years harbor a surprisingly similar microbiome that is
478 characterized by certain core community members (41). The relevance of the
479 concepts involved in retaining stability within microbial communities has been
480 recently outlined by Shade and colleagues (81). They identify interactions between
481 different bacterial strains and species as one important factor in maintaining
482 community stability. The response of the community to perturbation accordingly also
483 depends on the particular interspecies interactions, and cannot be predicted based
484 on the sum of individual species traits alone (81).
485 Studies have identified cooperation between microbial species as the interaction type
486 that drives a productive and stable microbiome, e.g. in the human gut (82, 83). This
487 view has been challenged by recent mathematical analyses (59) that predict
488 cooperation among microorganisms to indeed increase microbiome productivity but
489 to negatively affect microbiome stability. The counter-intuitive result that cooperation
490 between species is destabilizing is based on positive feedback loops that lead to
491 runaway effects (59). This means that unconstrained cooperation leads to an ever-
492 increasing abundance of the cooperating species, which in turn can result in the

collapse of competing populations and eventually in the destabilization of the whole community (84).

Until very recently models predicted that high species diversity hinders community stability (85, 86). This is in contrast to empirical observations where the opposite has been observed, e.g. in the human microbiome (87, 79). These models focused on species networks with a random distribution of interaction types (Box 2). Most recently however, in ecological network models, Foster and colleagues introduced negative-feedback loops by increasing the number of competitive interactions in the network (59). This resulted in a stabilizing effect on the community. These models predict that competition between various members of the bacterial community is the main factor for maintaining a stable microbiome.

Even though models are valuable for making predictions, tractable experimental model systems are needed to be able to test these. Concerning interactions within the bacterial microbiome, testing the aspects leading to stability is of great importance, as also pointed out by Fischbach and Segre (56). We are certain that the *Hydra* model will make a useful contribution in understanding host associated microbial communities, as we are currently collecting data on the strength and nature of the ecological interactions between its different microbial species (*Fig. 1*).

Box 2: Types of interactions between species

Interactions between organisms can generally be defined with the help of the 'intra-action compass' (88), which characterises all possible interactions among members of the same or different species. Species interactions (in microbial communities) can be driven by diverse features such as metabolism, social traits (production of public goods) or environmental factors, like spatial organization (89-92). There are six

519 different kinds of basal interaction patterns present in nature, which can be used to
520 describe the ecological interactions between members of two different (microbial)
521 species (for potential interactions within the metaorganism *Hydra* see Deines and
522 Bosch (24)). For the species involved, interactions can have a positive (+), a negative
523 (-) or no impact (0). When the interaction for the species involved is a win–win
524 relationship (+/+) it is known as cooperation (in metabolic-terms: syntrophy). Win-loss
525 interactions (+/-) are classical predator-prey relationships (in metabolic-terms: food
526 chain with waste product inhibition). The loss-loss relationship (-/-) describes
527 competition between species (in metabolic-terms: substrate competition).
528 Amensalism (0/-) is an interaction in which one partner is harmed without conferring
529 an advantage to the other (in metabolic-terms: waste product inhibition). In a
530 commensalistic relationship (0/+), one partner benefits without helping or harming the
531 other (in metabolic-terms: food chain). But also no interaction (0/0) can be found
532 between species (in metabolic-terms: no common metabolites) (93, 94, 92).
533 Disentangling the network of interactions between microbial species is challenging
534 but a combination of bottom-up and top-down approaches is available, ranging from
535 experimental (*in vivo*, *in vitro*) to *in silico* modelling approaches.
536
537
538 Another factor facilitating the stability of its microbiome is the host itself (59). Several
539 mechanisms have been identified by which a host may be able to suppress the
540 positive feedback between cooperating species and weaken their interaction. In the
541 following we summarize the available evidence from *Hydra* where the host shapes
542 the interactions between microbial species: First (i) regulation through the immune
543 response is dependent on the density of a particular microbial species. Observations
544 in the *Hydra* system where an increase in abundance of certain members of the

545 microbiome, i.e. Oxalobacteraceae and *Pelomonas sp.*, provoke a targeted immune
546 response (48, 95) is indicative of such as mechanism. Specifically have the host's
547 AMPs hydramacin and arminin been observed to increase in their expression levels
548 after the increase in abundance of the two microbial species. This could potentially
549 have a negative effect on the positive feedback loop between these two microbial
550 cooperating species, as AMPs are known to selectively target specific taxa, while not
551 affecting others (96). Nevertheless, the observation still needs to be experimentally
552 tested to confirm causality. Second (ii) spatial segregation reduces between-species
553 contact and so minimizes interactions. After microbes adhere to surfaces they start to
554 grow, divide, and interact with each other forming matrix-embedded communities,
555 termed biofilms. The structure of these communities can be either a disordered
556 mixture of strains or it can become highly structured such that the final community
557 contains large patches of single species (97). The same principles can be assumed
558 to apply for *Hydra's* ectodermal glycocalyx surface, a habitat for a complex microbial
559 community. Very recent findings provide the first evidence that *Hydra's* microbiome is
560 spatially structured. Augustin and colleagues (personal communication) show that a
561 specific host neuropeptide in *Hydra* leads to a spatial distribution along the body axis
562 of the main colonizer *Curvibacter sp. (Fig. 1)*. Third (iii) provisioning of carbon
563 sources via epithelial feeding minimizes cross feeding between microbes. For
564 humans it is well established that the gastrointestinal mucus layer not only limits the
565 contact between microbes and epithelial cells but also serves as a food source for
566 many gut bacteria (98). The types of modifications of mucins and the downstream
567 effects on community members are complex but it has been hypothesised that
568 carbohydrates play an important role in the interaction between host and microbes
569 (99). There is also evidence from corals that the mucus is used by commensal
570 bacteria (100), which strongly suggests that such metabolic interactions are also

present between *Hydra's* glycocalyx and its microbiota - an aspect that is currently under investigation.

5. Which role do viruses play in the competing interactions?

The freshwater polyp *Hydra* is not only associated with bacteria they feature a diverse eukaryotic viral community and bacteriophages. Eukaryotic viral community identified in *Hydra* affiliate to e.g. Phycodnaviridae, Herpesviridae, Baculoviridae and Poxoviridae (101). Viruses of these families are known to cause severe disease in a variety of different organisms including plants, vertebrates and invertebrates. Most of the recognized viral infections are acute viral infection with a rapid progression of disease, a restricted period of disease symptoms followed by a final clearance of viral infection by the host immune system. The host innate immune system is a fast defense mechanisms responding within the first minutes after viral infection. Pathogen associated molecular patterns (PAMPs) such as viral proteins, glycoproteins, RNA or unmethylated CpG in viral DNA are recognized by pattern-recognition receptors (PRR) e.g. RIG-1, NOD like receptors or TLPs leading to RNA synthesis of cytokines e.g. interferon a, and b TNF-a, IL-6, Il-12 and IFN-Y (102). Cytokines stimulate the production of antimicrobial peptides. Antimicrobial peptides are important effectors of innate immune system regulating bacteria, fungi but also viruses. Antimicrobial peptides such as defensins can either act directly on viruses or indirectly by affecting target cells (103). However, not all viral infections are entirely cleared. Some viruses evade the host immune defense and establish persistent infections e.g. (humans varitella-zoster virus, measles, HIV, cytomegalovirus). These infections can be chronic with a continuous proliferation of

597 virions for a long period or viruses switch from a lytic to a latent state were their
598 nucleic acid is integrated into the host genome. Virome sequencing and increase of
599 genomic data revealed that persistent viral infections are common and present in all
600 domains of life. Also *Hydra* is associated with a species-specific persistent viral
601 community that can be expected to modulate *Hydra's* functions.
602
603
604 ***Host-virus interaction***
605
606 In the same way host has evolved to control viral infections viruses have developed a
607 variety of different mechanisms to manipulate their host. For this reason host-virus
608 interactions have a profound impact on cellular pathways and influence the host
609 metabolism. Several viruses are known to stimulate host interleukin pathway (human
610 immunodeficiency virus HIV, hepatitis C hepatitis B) or produce their own viral
611 orthologue (herpesviruses and poxviruses). Interleukins are crucial for many viruses
612 to establish persistent infections and blockage of this pathway facilitates virus
613 clearance. Consequently different aspect of the chemokine system have been
614 exploited by viruses and viruses encode proteins with homology to chemokines and
615 chemokine receptors (104). Host-viral interactions are not only present during acute
616 infections. Most of the viruses remain active throughout latency. Epstein-Barr virus
617 latency persist in B cells, epithelial cells and T-cells. It remains active and expresses
618 genes manipulating cellular gene transcription, induces G1 arrest, chemokines,
619 promotes cell proliferation, activates NF-kB, p38 and other pathways, blocks antigen
620 dependent signaling, suppress differentiation, promotes epithelial cell spreading and
621 inhibit apoptosis (105). *Baculoviruses* that were also found in the virome of *Hydra*
622 and replicate within *Hydra* tissue (*Fig. 3*) are another well-studied example of how

623 viruses manipulate their hosts. Already during *Baculovirus* latency a subset of genes
624 are transcribed and interact with cellular pathways. A variety of immediate early,
625 early and late gene products manipulate cell-cycle arrest, remodel cytoskeleton,
626 metabolism, immune response, inhibit apoptosis (106). Similar interactions between
627 host and viruses have been reported for herpesviruses. Herpesviruses are already
628 associated with basal metazoans *Hydra* and corals (100, 107). Along the
629 phylogenetic tree herpesviruses are present among others in molluscs (108), fish
630 (109-111), birds (112) to humans. This ancient association between herpesviruses
631 and metazoans has coevolved a strong interaction of herpesviruses and their hosts
632 (113). In *Hydra* and corals herperviruses are one of the most abundant viruses
633 representing more than 50% of the associated eukaryotic viral community (100, 107)
634 and there is first evidence that they play a beneficial role in sustaining coral health
635 (114).
636 Viral induced reconstruction of cellular functions may affect only a small subset of
637 cells and remain locally controlled with little impact on the entire individual. Severity
638 of viral infections and the switch from latent to lytic viral replication highly depends on
639 the type of virus and environmental factors that influence virus-cell interactions (115).
640 Oncogenic viruses are one example that virus induced cell manipulations can have
641 severe consequences for its host (116). However, not all viruses are negative and it
642 can be expected that most of the viruses are neutrally associated with their host or
643 even have a positive impact. In *Hydra* we identified a diverse viral population, which
644 has not been recognized so far as *Hydra* is presumed to be immortal under constant
645 laboratory conditions and does not show any signs of disease symptoms. However,
646 under temperature stress condition we can induce some shifts in the natural viral
647 community composition leading to e.g. an increase of *Baculoviruses*. Persistent viral
648 infections that are sensitive to environmental stress might function as selective

649 regulators within the diverse cell population. In latent virus infected cells that are not
650 able to compensate for environmental imposed alterations of viral-cell interaction the
651 viral lytic lifecycle is induced finally terminated by the death of the cell. Thus, viruses
652 are selective and able to function as regulators within cell populations with a positive
653 impact on its host can be illustrated by Oncolytic viruses (117, 118). Several viruses
654 are able to infect cancer cells and replicate within these cells. Although oncolytic
655 viruses can infect normal cells cancer cells are due to several different defects
656 regarding cellular signaling and stress response beneficial for viral replication (117,
657 118).
658
659 ***Virus-virus interaction***
660
661 Viral infections do not only affect the host they also impose a strong impact on other
662 viruses. Virus-virus interaction can be directly mediated through viral genes and gen
663 products or indirectly through viral induced alteration of the host, detailed reviewed in
664 DaPalma and colleagues (119). Being associated with a diverse viral community like
665 *Hydra*, implies complex virus-virus interaction already within the host associated viral
666 community. Secondary invading viruses from the surrounding water encounter the
667 present viral community that have already coevolved with its host and established a
668 homeostatic relation or balanced association with their host cells. This viral related
669 reprogramming of host cells shape the present cell population and can induce
670 resistance to subsequent infection by similar viruses (superinfection exclusion) (120).
671 Environmental stress can destabilize natural host viral homeostasis, which may
672 facilitate secondary invasion by tissue damage and loss of barrier functions (101,
673 107). There are also several examples in the literature of cooperative virus-virus
674 interactions. In these cases viral infection depends on viruses that have previously

675 infected and modified the host cell in the way that a secondary virus is able to infect
676 (e.g. human retrovirus) (121). On the other hand secondary viral infection can also
677 transactivate latent viruses of the host. Transactivation of latent viruses can be
678 triggered directly by gen products of another heterologous virus or indirectly by
679 changing the expression of host genes. Most of these interactions within the viral
680 community occur on a cellular level and only affect a subset of the cell population
681 without causing any visible disease symptoms. Double infections are then recognized
682 e.g. if they cause an acceleration of disease. For this reason most of these
683 interactions remain unseen. However, increasing number of reports illustrating the
684 complexity of viral communities associated with metazoans point to complex viral-
685 viral interactions within metaorganisms. Multiplicity of viral infections of one individual
686 implicate an increased chance that co-infections appear within one cell. This may
687 lead to a diversification of viruses by genetic recombination of parental viruses,
688 generation of pseudotyped viruses or to the integration of e.g. retroviruses into the
689 genome of other viruses.
690
691 ***Virus-bacterial interaction***
692
693 Viral infections often lead to the debilitation of the host facilitating secondary
694 infections by bacteria. This can be due to disturbance of barrier functions, such as
695 virus induced cell death or change of host cell membranes leading to an increase of
696 bacterial attachment. Viral alteration of the immune system reduced expression of
697 antimicrobial peptides or down regulation of TNF-a (122, 123). While these inside-out
698 regulations implies an already established virus-host association, novel invading
699 viruses have to cross not only natural barriers, such as mucus layers, glycocalyx and
700 cell membranes of the host (*Fig. 1*). In most organisms and also in *Hydra* these

surfaces are already colonized by commensal microbiota. Host bacterial but also bacteria-bacteria interactions shape the surface environment, which can highly impact the infectivity of eukaryotic viruses (124). While there are several examples of probiotic bacteria featuring antiviral activity it becomes more and more apparent that these effects are most likely mediated indirectly by bacteria induced modulation of the host immune response (125). In general the presence of commensal microbes leads to an upregulation of immune responses suggesting germ-free individuals to be more susceptible for viral infections due to a compromised immune system. However, this causal link is only true for some viruses. As viruses have coevolved with its host and its associated microbes, infectivity of several viruses highly depends on the presence of the associated microbial community. For example transmission of retrovirus depends on the commensal microbiota to induce an immune evasion pathway (126). Poliovirus infection depends on lipopolysaccharides (LPS) produced by its host associated bacteria protecting the virion from inactivation and enhances viral attachment to cellular receptor (127). This and several additional examples of virus-bacteria interaction are reviewed by Robinson and Pfeiffer (128).

Phage-bacterial interaction

In the aquatic environment *Hydra* is permanently exposed to bacterial colonizers as well as to phage infections that interfere with the host specific microbiota. Preventing foreign bacteria from settlement and control phage infection are beside the internal regulation of the host associated bacterial community important for the maintenance of host specific bacterial community composition. Phages are compared to bacteria highly abundant (129, 130) and strong regulators within bacterial populations (131-133). Maintaining a stable microbiota implies strong defense mechanism against

phage infections. As phages evolve rapidly bacteria have developed a brought range of strategies to protect themselves from infection. Mechanism to control phage infections have been reviewed in detail (134, 135) and can be grouped into (i) preventing phage attachment by blocking phage receptors, excretion of extracellular substances or production of competitive inhibitors; (ii) blocking DNA entry; (iii) cutting phage nucleic acid by restriction modification or Crisper-Cas system; (iv) abortive infection; (v) assembly interference; (vi) blocking phage DNA replication by BREX system (136) and (vii) arbitrium communication system (137).

Living associated with *Hydra*, embedded into the mucus-like layer of *Hydra's* glycocalyx (45) could be another, so far neglected mode of protection of bacteria against phage infections. An accumulation of virus like particles (VLPs) at the surface of mucus layers have been reported for different organisms and it has been shown, that phages bind to mucus glycoproteins via Ig-like proteins domains on phage capsids (138). While this observation can be interpreted on one hand as host derived protection of its associated bacteria against phage infection, the authors hypothesize that the presence of phages at the outer mucus layer could serve as a non-host derived immune defense. While the function of phages within host derived mucus layers is still in its infancies more research has been conducted on bacterial biofilms. Similar to bacterial communities that live within host derived mucus layer, biofilm bacteria live in a three dimensional matrix of exopolysaccharides (EPS). Living within a biofilm not only protects bacteria from physico-chemical stress, it also protects bacteria from phage infections. Some phages have adapted to this environment and carry polysaccharase to actively degrade EPS enabling attachment to bacterial surfaces for infection (139). Analogous to biofilms phage invasion of the mucus-like layer of *Hydra* can be expected to afford evolutionary adaptation to overcome this

752 natural barrier. Nevertheless, bacteria living in the periphery are more likely to get
753 infected then those deeper inside.
754 Recently we have analyzed the phage community composition of different *Hydra*
755 species and revealed that *Hydra* is associated with a species-specific phage
756 community (100). It can be expected that the phage population is composed of
757 transient phages by meaning phages that originate from the surrounding water and
758 adhere to *Hydra's* surface or infect *Hydra's* associated microbiota and of a resident
759 phage community. First insides into the resident phage population we gained by
760 simple bacteria-bacteria interaction experiments between the most dominant
761 bacterial colonizer of *Hydra Curvibacter sp.* and the second abundant bacteria
762 *Duganella sp.* in vitro (67). The observed frequency dependent growth rate was not
763 explainable by only two interacting bacterial strains and a phage as third player was
764 predicted. Screening the genome of both bacteria revealed the presence of a
765 prophage signature in the genome of *Curvibacter sp.* Finally we were able to
766 reactivate the temperate phage of *Curvibacter sp.* and could show that this phage is
767 able to cross-infect *Duganella sp*.. The presence of hidden prophages within *Hydra*
768 associated bacteria directed us to screen our bacterial culture collection for the
769 presence of lysogenic phages and we found that approximately 50% of *Hydra*
770 associated bacteria carry a prophage in their genomes. In this lysogenic state of
771 bacteriophage lifecycle phage DNA is integrated into the bacterial genome and is
772 replicated passively during bacterial cell division. Analogue to latent eukaryotic viral
773 infections lysogenic phages are transcriptional active and able to modulate their
774 bacterial host e.g. metabolism, virulence factors, stress tolerance (140). This
775 lysogenic conversion increase the genetic repertoire of the bacterium by horizontal
776 gene transfer but may also change or shape host bacterial interactions, e.g. by
777 modifying outer membrane lipopolysaccharides (141). Carrying a prophage can be

778 beneficial as it protects the bacterium from similar phage infections by superinfection
779 exclusion. Switching from a lysogenic to a lytic lifecycle can be advantages for the
780 bacterium as their phages can serve as weapon against competitors. This in turn can
781 have regulatory functions within the *Hydra's* associated bacterial community and
782 prevent bacterial invasion from the surrounding environment. Prophages of *Hydra*
783 associated bacteria can be reactivated and switch to a lytic replication. This switch is
784 driven by different environmental factors but also depends on the state of bacteria
785 growth rate, which emphasis a potential link between nutrition and both function and
786 stability of the associated microbiota. Thus, prophages can be induced under
787 environmental stress conditions it can be expected that *Hydra*-bacteria-phage
788 interactions are dynamic systems, which have to be continuously balanced and
789 brought into equilibrium to finally maintain metaorganism homeostasis. Moreover it
790 can be speculated, that host factors, such as antimicrobial peptides can also interfere
791 with the lysogenic state of bacteria and are able to induce phage replication (142).
792 Host intervention in bacterial phage interaction might be one potential mode to fine
793 tune bacterial-phage interactions and to control its specific microbes by using
794 prophages as internal regulators. On the other hand proliferation of phages by the
795 host specific bacterial community could help to defend against secondary bacterial
796 infection according to the bacteriophage mediated immunity proposed by Barr and
797 colleagues (138, 143).
798
799
800 **Conclusion**
801 How a metaorganism is established during ontogeny and remains in balance
802 over time is a critical question regarding many aspects of life. Here we propose
803 that *Hydra* is an informative model system to explore how the microbiome and

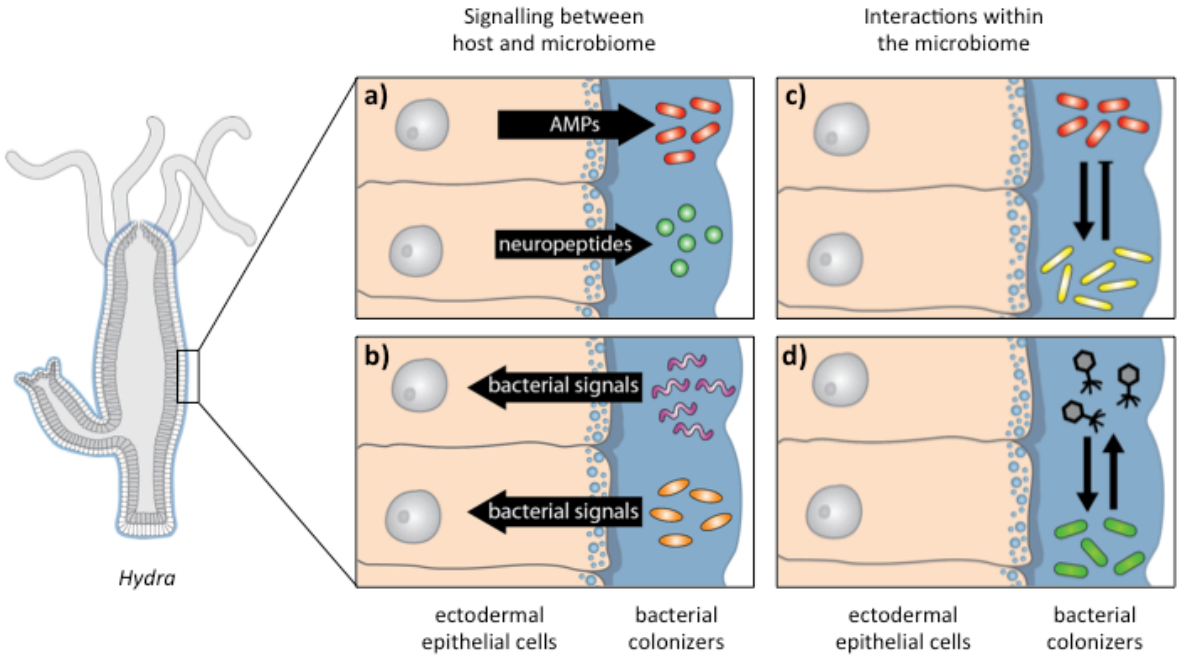
virome is established and maintained under different environmental conditions. Ontogeny is a process in which the associated partners bacteria, phages and viruses are exposed to a consecutive pattern of a newly shaped host environment. Varying environmental conditions during development can re-shuffle complex interactions within the holobiont assemblage, which form and prime the metaorganism. We propose that not only the holobiont composition, but even more the network of interactions that have been established within the holobiont during ontology contribute to the stability of the metaorganism. Development of a metaorganism continues throughout the lifespan of the host allowing a continuous fine tuning of the established network under varying environmental conditions ensuring the function and homeostasis of the metaorganism.

Acknowledgments

The authors apologize that, because of the selective focus, many interesting investigations and reviews were not included. The authors' work related to this topic was supported in part by grants from the Deutsche Forschungsgemeinschaft (DFG), the Collaborative Research Center (CRC) 1182 ("Origin and Function of Metaorganisms") and grants from the DFG Cluster of Excellence programme "Inflammation at Interfaces". PD acknowledges funding from the European Union's Framework Programme for Research and Innovation Horizon 2020 (2014-2020) under the Marie Skłodowska-Curie Grant Agreement No. 655914 and from the Deutscher Akademischer Austausch Dienst (DAAD). TL acknowledges funding from the Volkswagen Foundation (Funding program "Experiment! – *In search of bold research ideas*"). TB gratefully appreciates support from the Canadian Institute for Advanced Research (CIFAR).

836 **Figures**

837 Figure 1.



838

839 **Modes of signalling and interactions in *Hydra*.** (a). Antimicrobial peptides (AMPs)
840 and neuropeptides produced by the host modulate the host associated microbial
841 community. (b). Microbially produced metabolites act as signalling molecules on
842 distant targets such as the nerve net. (c). Microbe-microbe interactions can have a
843 positive, negative, or no impact on the species involved. These ecological
844 interactions are key components of a stable microbiome. (d). The viral community
845 may contribute to maintaining microbial population equilibrium and community
846 resilience.

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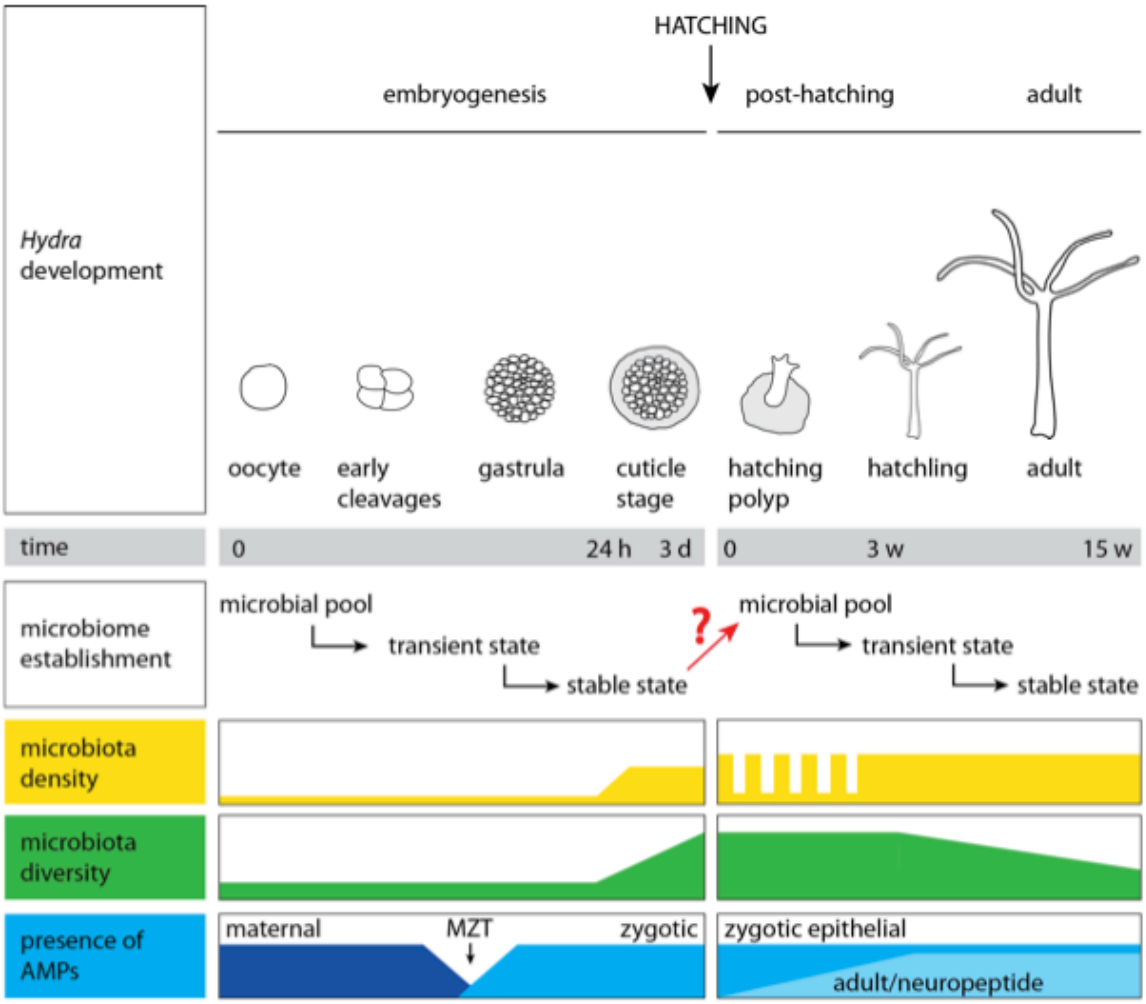
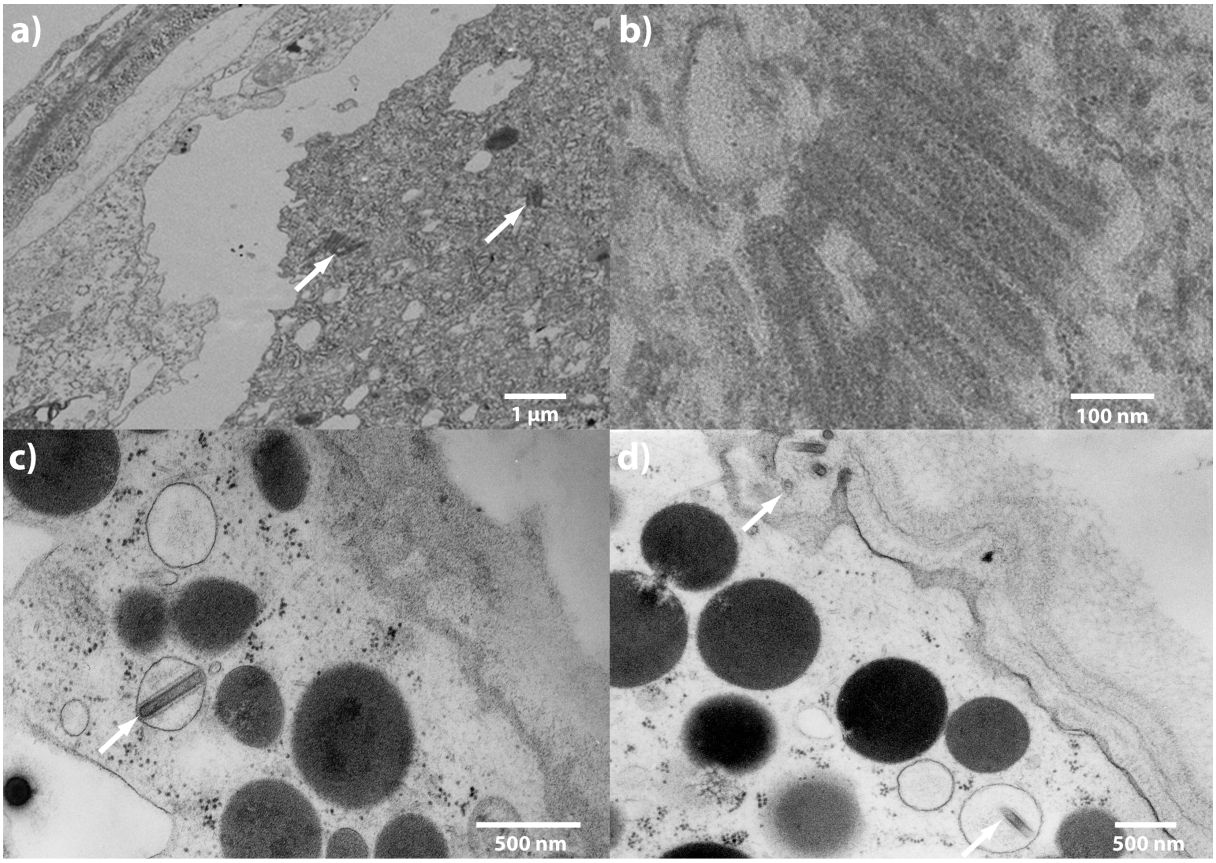
Figure 2.



Bacterial colonization of *Hydra* during embryogenesis and microbiome progression from post-hatching to the adult polyp. Pre-hatching and post-hatching developmental stages are characterized by the expression of specific antimicrobial peptides (AMPs) mediating host-microbe homeostasis. The maternal-zygotic transition (MZT) is the most critical phase during embryogenesis and coincides with the transition from maternally to zygotically produced AMPs. These changes go in hand with changes in microbiome density and diversity (48, 71). Community assembly during embryogenesis and post-hatching follows specific trajectories but it is so far not clear whether the cuticle stage microbiome serves as a microbial pool for the hatching polyp.

865 Figure 3.



866

867 **Baculoviral replication in *Hydra*.** Transmission electron micrographs of ultrathin
868 sections of *Hydra* negatively stained with uranyl acetate illustrating the presence of
869 *Baculoviruses* in *Hydra* tissue. *Baculoviruses* are replicated within *Hydra* cells (a&b).
870 Virons are transported in vesicles (c) and released through the ectodermal cells (d).

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875

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