Opinion

Host, Symbionts, and the Microbiome: The Missing Tripartite Interaction

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Symbiosis between microbial associates and a host is a ubiquitous feature of life on earth, modulating host phenotypes. In addition to endosymbionts, organisms harbour a collection of host-associated microbes, the microbiome that can impact important host traits. In this opinion article we argue that the mutual influences of the microbiome and endosymbionts, as well as their combined influence on the host, are still understudied. Focusing on the endosymbiont Wolbachia, we present growing evidence indicating that host phenotypic effects are exerted in interaction with the remainder microbiome and the host. We thus advocate that only through an integrated approach that considers multiple interacting partners and environmental influences will we be able to gain a better understanding of host–microbe associations.

Interacting Entities

Arthropods commonly host a wide variety of microbes (see Glossary), some of which live within a host in a close and long-term biological interaction. Such endosymbionts can exert effects on the host ranging from positive interactions (mutualistic, i.e., providing benefits [1]) to negative interactions (parasitic, i.e., imposing substantial costs [2]). Thus, endosymbionts are important modulators of host phenotypes, providing heritable variation upon which natural selection can act [3,4].

Historically, symbiosis research has focused on binary interactions between hosts and individual endosymbionts. In recent years this view was broadened to include all microbes that copiously colonize animals, the so-called microbiome, as they are additional important modulators of host traits (Box 1). Due to the historical focus on binary interactions, comparatively little is known about interactions between microbes within the microbiome and how these interactions impact the host [5]. A more holistic approach towards the multitude of interactions is, however, needed for a better understanding of the variety of mechanisms by which microbes drive animal health, development, and evolution [6]. This is especially true as symbionts are part of a complex ecosystem including host, symbiont, microbiome, and their environment. Here symbionts, host, and the remainder microbiome interact with each other, but are also influenced by free-living microbial communities and environmental conditions, for example, temperature, diet, as well as other organisms (Box 2). Focusing on only one type of interaction, that is, between host and symbiont or between host and the microbiome, under artificial conditions that do not reflect the potential influence of the environment (Box 3) will provide an incomplete picture of host–microbe interactions.

In this opinion article, we argue that an important area for future research relies on disentangling how endosymbionts, the remainder microbiome, and the host interact with each other and how their environment is shaping these interactions (Figure 1). Note that we consider symbionts as a separate microbial entity due to historical focus on binary interactions of symbiont and host. With
Box 1. The Host as an Ecosystem

Historically, symbiosis research in insects has focused primarily on binary interactions between hosts and individual endosymbionts [3,33], and therefore observed phenotypic effects were attributed to the single symbiont. This binary point of view has been challenged in recent years. The ‘microbiome revolution’ [54] of the past 10 years revealed that all animals are copiously colonized by microorganisms, collectively called the microbiome, of which the symbions are part. During this revolution it was realized that, similar to single symbions, the microbiome can also impact important host traits [17,55,56] and thus influence the ecology and evolution of their hosts [57], acting as an extended genome of the host, the hologenome [58]. Therefore, it has been proposed that the host itself should be viewed as a complex ecosystem, in which not only single symbions interact with the host, but also the microbiome interacts with symbions and hosts [37,57]. An additional scale of complexity has recently gained attention, namely that host–microbe associations are also part of a wider microbial community maintained by transmission between individual hosts and dispersal between host-associated and free-living microbial communities [8,28]. In 2017, Carrier and Retzel [59] introduced the idea of a ‘host-associated microbial repertoire’, which is the sum of microbial species a host may associate with over the course of its life. Due to this plasticity, the microbiome genome could allow hosts to adapt and survive under changing environmental conditions, thus providing the time necessary for the host genome to adapt and evolve [60]. From this it becomes clear that one host can harbour a diverse and interacting microbial community, whose components potentially compete for space, energy, and resources, ultimately influencing the condition of the host by conferring multiple detrimental, neutral, or beneficial effects [61]. Therefore, a more holistic approach in studying the interaction between the different partners is needed.

this separation we can highlight the differences between both, but in fact the microbial symbions are part of the microbiome and, as such, should be studied together. We postulate that phenotypic effects of symbions are modulated by other microbes, the host, and the tripartite interaction between them. Drawing on the wealth of information on the endosymbiont Wolbachia, we discuss studies that embraced this holistic view. We argue for re-investigating well known symbioses with respect to the interactions with other microbes, reviewing studies that indicate that symbioses are more influenced by other partners than the host itself. Throughout this opinion article we advocate the importance of applying this holistic view to gain a better understanding of how symbions and microbiome interact with each other and the host and how these interactions shape host fitness.

Symbions, the Sole Manipulator?

In recent years researchers started to investigate microbe–symbiont interactions in model organisms such as the fruit fly, Drosophila melanogaster [7], the parasitoid wasp Nasonia [8], and mosquitoes as vectors of important human diseases [9]. Additionally, projects like the

Box 2. Factors Influencing Host Microbiome Specificity

Host–microbe interactions are shaped by a multitude of factors, that is, factors associated with the host such as immunity [62], phylogeny [63], host population background [64], physicochemical conditions in the insect habitat (e.g., gut pH, oxygen tensions), and structural filters in the gut [65], but also environmental factors such as diet [66] and temperature [67]. Abiotic factors do have a crucial effect on microbes and the host, and therefore on their interactions. External environmental conditions significantly affect the infective states of hosts, including the density of the endosymbionts inhabiting the host body, for example, high temperature [4,25,26] with occurring seasonal changes of symbiont density (such as Wolbachia [9,26,27]).

In addition, these associations are also part of a wider microbial community maintained by transmission between individual hosts and dispersal between host-associated and free-living microbial communities [8,28,59]. While understanding the factors that affect the composition and abundance of the microbiome is expanding, there are still many unanswered questions regarding microbiome assembly and maintenance. Exposure to environmental microbes has undoubtedly a major influence on the microbial communities of an organism [42], as metacommunity studies revealed that microbial communities associated with different interaction partners (species) differ in terms of composition and abundance, but shared microbes among the macro-partners [30,31]. Unravelling the role of the environment in shaping the host-associated microbiome (including symbions) is crucial to place the specificity of interactions in an evolutionary context, for instance, by understanding whether deterministic processes lead to the selection of the horizontally transmitted microbes.

Glossary

**Ecosystem**: the complex network of living organisms, their physical environment, and their interactions in a particular unit of space. In our context a host, its associated microbiome, and all potential interactions among living organisms and with environmental conditions.

**Endosymbionts**: microbial associates living within the body or cells of another organism (host).

**Hologenome**: the sum of the genetic information of the host and its microbiome.

**Horizontal transmission**: transmission of microbes between host individuals, species, or by acquiring free-living microbes from the environment.

**Host**: an organism in which an endosymbiont or microbiome lives.

**Host-microbe interactions**: interactions between any microbial species or microbial communities (either a symbiont or part of the remainder microbial community) and a host.

**Hub species**: microbial species that are strongly interconnected by several links within a network and play an important role in community functioning and/or stability. Abiotic factors and host genotype can directly act on hub species, thus spreading the effects to the whole microbial community.

**Metacommunity**: a set of interacting communities that are regulated by processes such as dispersal, extinction, and re-colonization.

**Microbes**: microscopic organisms, including bacteria, fungi, protozoa, and viruses.

**Microbiome**: a community of microbes that inhabit a particular environment.

**Parthenogenesis**: clonal reproduction, in which an unfertilized egg develops into a new individual.

**Phenotype**: the set of observable characteristics of an individual resulting from the interaction of its genotype with the environment.

**Phenotypic effects**: changes in a phenotype caused by an external influencing factor, here symbions.

**Remainder microbiome**: the microbiome excluding the symbiont under investigation. Note that we consider symbions here as a separate microbial entity, due to historical reasons, and to highlight the differences between both, but in fact they are a part of the microbiome and, as such, should be studied together.
Box 3. The Importance of Laboratory versus Field Studies

Given the strong influence of environmental factors (Boxes 1 and 2) on host–microbe interactions, the transition from laboratory studies to field studies might be difficult [59]. Laboratory settings potentially restrict the full spectrum of host–microbe associations compared with the natural setting where these associations have evolved [62]. Thus, it may limit the interpretation of the functional roles microbes play in host biology [59; Box 2].

A good example of this is the Wolbachia-mediated inhibition of dengue virus. Under laboratory conditions it was indicated that the microbiome composition of the mosquito Aedes aegypti is not critical for inhibition [40]. However, when released into the wild, the picture became more complex. Wolbachia, when introgressed into different genetic backgrounds, increased the mean and the variance in mosquito susceptibility to dengue infection [41]. While the respective impacts of these factors are not easily disentangled, similarly complex multifactorial patterns likely underlie many host–microbe associations under ecologically realistic conditions. Given that Wolbachia appears to modify host susceptibility to a broad spectrum of pathogens [34,68], reliable predictions of invasiveness and vectorial capacity of transinfected mosquitoes require an informed account of natural mosquito pathogens and their interplay with Wolbachia [41]. A first step to get a more complete picture of the symbiotic effects of Wolbachia is to investigate these complex interactions simultaneously in the laboratory and field. In addition, a beneficial approach would be to mimic environmental factors, biotic and abiotic, in the laboratory. Microbiome and symbiont manipulation are often achieved through the use of antibiotics. However such approaches may have several overlooked side effects. First, antibiotics may affect different components of the microbiome differently and hence alter the interaction networks. Second, results obtained in the absence of (parts of) the microbiome tell only one side of the story, as microbe–microbe interactions might modulate host response. Hence, in order to unravel the interactions and impact of host phenotype, host recolonization by a variety of well characterized microbes, or whole microbial communities, is required. Also the enrichment of the microbial diversity, mimicking possible biotic environmental influences such as transmission of free-living microbial communities, might be worthwhile. Although these manipulations can provide only a mechanistic understanding of the tripartite interaction, and may not be translatable to field settings, they nevertheless are an important first step in gaining a better understanding of host–microbe interactions.

Symbionts and the remainder microbiome can influence each other and, by doing so, potentially shape their effects on the host phenotype. For example, the microbiome can be a potential barrier to transmission of heritable symbionts through competitive exclusion of maternally inherited bacteria, as shown for the American dog tick Dermoecentor variabilis [11] and the fruit fly D. melanogaster [12]. On the other hand, symbionts and host can, together, control and shape the microbiome as shown in Lepidoptera [13], D. melanogaster [7,14], and the mosquito Aedes aegypti [15].

These interactions between the different members of the microbial community within a host can either be direct, via competition for resources and space [12], or indirect, via the induction of a general immune response [16,17]. A direct competition has been hypothesized for the protective phenotypes induced by the endosymbiont Wolbachia against pathogens in Drosophila and Aedes, resulting in abundance-dependent protection [18,19]. Competition for resources or space between Wolbachia and other bacteria is also likely for the terrestrial isopod Armadillidium vulgare. In this pill bug, total bacterial loads increase in some, but not all, tissues of Wolbachia-infected individuals [20], and the presence of Wolbachia decreases the abundance of bacterial phylotypes [21]. The nutritional mutualism in the bed bug Cimex lectularius is an other example for direct interactions. The exchange of genetic material between Wolbachia and other symbionts (likely either Cardinium or Rickettsia) conferring the bed bug enabled Wolbachia to become an obligate symbiont providing B vitamins to the host [22,23]. Indirect interaction between the different members of the microbial community of a host has also been found. In bumble bees (Bombus terrestris), variation in gut microbiome seems to drive the general defence against parasites and with this the evolution of gut parasites by interactions with the remainder microbiome as well as
with host genotypes [17]. Similarly, in ticks (Ixodes scapularis), parasites induce the expression of specific glycoproteins, which alter the host microbiome to their advantage, that is, to promote infection [16]. These studies highlight the complexity of the tripartite interaction between host species, microbiome, and symbionts, across different hosts, and foster the development of a framework in which interactions, host phenotype, and environment are jointly explored.

The Environmental Component

The environment influences microbes within a host and thus potentially their interactions as well as their effect on host phenotype. For example, it is known that the abiotic environment, that is, temperature, affects symbiont density [4,24]. For instance, a reduction, or elimination, of Wolbachia – due to high temperature – was found for D. melanogaster, mites, and other species [4,24–26]. In line with this, seasonal changes in Wolbachia density were observed in Lepidoptera [26], mosquitoes [9], and other blood-sucking arthropods [27]. In mosquitoes, high temperature caused a reduction in Wolbachia density and a concomitant greater host susceptibility to viruses [9]. Also, the biotic environment could potentially influence within-host microbe–microbe
interactions through horizontal transmission of microbes from free-living microbial populations \[28\] (Box 2). This could lead to microbial community shifts and therefore changes in microbe–microbe interactions, potentially influencing host fitness. These interactions are defined by metacommunities, local communities linked by dispersal, but also extinction and recolonization of potentially interacting species \[29\]. Thus, local communities are influenced by processes operating at metacommunity level \[26\]. The few studies that investigated the influence of metacommunities discovered that the microbial communities associated with different interaction partners, for example, plants and insects, shared microbes \[30,31\], such as vertically transmitted symbionts from the genera *Wolbachia*, *Rickettsia*, and *Spiroplasma* \[30\].

As phenotypic host effects of a symbiosis are closely tied to interactions with the remainder microbiome and environmental factors, biotic and abiotic, the absence of an integrative approach might mask the mechanistic interpretation of the data, leading to inconclusive results. Taking *Wolbachia* as model, in the next section we provide a brief introduction of what is known for *Wolbachia*–microbe interactions and two examples in which a deeper understanding of the complex interactions between hosts, symbionts, and the microbiome could explain discrepancies. The first one is of great relevance for human health and refers to investigations on the vector-competence of *Wolbachia*-infected mosquitoes and their role in the transmission of human pathogens. The second example refers to the reproductive manipulation by *Wolbachia* in several arthropod species, and takes a more evolutionary perspective.

**Interactions between the Endosymbiont *Wolbachia* and Other Microbes**

*Wolbachia* in Interaction: Known Facts

The endosymbiont *Wolbachia* – one of the most widely distributed symbionts worldwide, infecting an estimated 40% of terrestrial arthropods \[32\] – is a strong manipulator of a wide range of host traits \[33\]. It gained specific interest due to its protection against various viruses in naturally infected fruit flies \[34\] and its capacity to reduce the density and transmission of pathogens in mosquito species \[35,36\].

In contrast to long-held beliefs, *Wolbachia* is not restricted to host germ-line cells and reproductive organs, but is present in cells throughout somatic tissues and even in the gut lumen of some insects and their faeces \[37,38\]. Thus, direct interactions with other microbes of the host, or indirect interactions via the hosts’ immune system, are likely. Direct interactions between *Wolbachia* and other microbes have been observed in fruit flies. A coinfection with the endobacterium *Spiroplasma* reduced *Wolbachia* density, while *Spiroplasma* numbers remained unaffected by the presence of *Wolbachia* \[12\]. The investigation of the effect of *Wolbachia* infection on the composition of the gut microbiome in *D. melanogaster* showed an even more complex picture. Here the presence of *Wolbachia* is a significant determinant of the overall composition of the gut microbiome. Interestingly, this was caused neither by a direct interaction between *Wolbachia* and the gut microbiome – as *Wolbachia* is absent from the gut lumen in the fly – nor by indirect modulation through the activation of the fly’s immune system by *Wolbachia* \[7\]. This highlights the importance of considering a multitude of possible interactions between microbes and between microbes and the host in studies of the dynamics and effects of *Wolbachia* infections.

**The Quest for Disease Eradication**

An integrated approach, considering multiple interactions between microbes and between microbes and hosts, is especially important when developing microbe-based disease vector-control strategies. *Wolbachia* is currently being developed as a novel arthropod-borne disease control agent (http://www.elimatedengue.com). Hereby, a successful transmission and stable
infection are important factors, as the efficiency of the control agent relies on it. Under laboratory conditions, the native microbiome of Anopheles mosquitoes was found to affect vertical transmission of Wolbachia through antagonistic bacterial interactions between the bacterium Asaia and Wolbachia [39]. Similar antagonistic microbe interactions were found in a survey of various mosquito species in Canada, with the presence and abundance of Wolbachia fluctuating over season, as well as with the presence of the bacteria Asaia and Pseudomonas [9]. This suggests that, in addition to environmental effects, the interaction of Wolbachia with other microbes may explain some of the variation in vector competence of mosquitoes. In contrast, a stable infection with Wolbachia in laboratory-reared mosquitoes (A. aegypti) had only few effects on the microbiome. Moreover, significant changes in the microbiome composition did not affect the dengue-virus-blocking phenotype caused by Wolbachia infection in this host [40]. However, analyses of A. aegypti transinfected with Wolbachia, released in the field in Brazil and Vietnam to inhibit the dengue virus, revealed that Wolbachia increases susceptibility of mosquitoes to dengue infection. This contradicting result was due to the wide variability in exposure doses of Wolbachia naturally experienced by mosquitoes [41]. The authors concluded that reliable predictions of vectorial capacity of transinfected mosquitoes require an informed account of mosquito pathogens and their interplay with Wolbachia. Additionally, recent interaction networks, looking at co-occurrence and coexclusion of microbes, established for several mosquito species (laboratory versus field) revealed that Wolbachia is a highly interconnected taxon, mostly coexclusionary with other bacteria [42].

The mosquito studies indeed show that the abundance and effect of Wolbachia are closely tied to the remaining microbiome. This highlights the importance of considering the composition of the microbiome and host genetic background in studies investigating phenotypes induced by Wolbachia and when formulating microbe-based disease vector-control strategies. In line with that, assessing the involvement of microbe–microbe interactions within a host, and how they are influenced in the field, due to biotic or abiotic factors, is critical as it may affect the efficiency of Wolbachia-mediated manipulations (Boxes 2 and 3).

The Joint Reproductive Manipulation of Wolbachia

The endosymbiont Wolbachia is especially well known for its four distinct reproductive phenotypes that promote its own vertical transmission from mother to offspring [43]. There is growing evidence that the reproductive manipulation by Wolbachia is not only exerted by the endosymbiont alone but in interaction with other microbes, that is, the microbiome of the host, other symbionts, or the host itself. Wolbachia has repeatedly been reported to cause different phenotypes, either in experimental settings, when transferred between hosts, for example in Lepidoptera [44] or Drosophila sibling species [45], or naturally over evolutionary timescales, for example, in moths and fruit flies [46]. Additionally, many species show geographical variation in symbiont prevalence, including Wolbachia with a lower presence in warmer regions [4], as for example reported for many species infected with parthenogenesis-inducing Wolbachia [47]. The causes for this distributional pattern in prevalence remain speculative, but a possible explanation is that it is driven by variation in microbial communities of host populations in interaction with their abiotic environment (Box 2).

Another line of evidence indicating a modulating role of the microbiome in reproductive manipulation by Wolbachia comes from studies investigating Wolbachia abundance (titre) in a host. The efficiency and phenotype of reproductive manipulation can depend on a threshold of Wolbachia titre, that is, a minimum number of bacteria is required for exerting the manipulative action. A low titre can lead to a switch of the Wolbachia-induced phenotype in Drosophila bifasciata [24], or to changes in the efficiency of parthenogenesis induction in the parasitic wasp Asobara japonica.
Together, these examples illustrate that Wolbachia may be a potent manipulator of host reproduction, not in isolation but rather in interaction with the host genome and the remainder of the microbiome, and in addition influenced by interaction with the environment. By shifting the focus away from Wolbachia as the only manipulator it becomes clear that manipulation of a host phenotype is likely not caused by only a single microbe (Wolbachia), but is also strongly influenced by interaction with other microbes, and by the host genotype itself.

**Concluding Remarks**

Throughout this manuscript we have pointed out growing evidence that host phenotypic effects, such as reproductive manipulation by the endosymbiont Wolbachia, are exerted not only by an endosymbiont alone but in interaction with other microbes. This, and other, examples call for an integrative approach in studying host–microbe associations, including host gene expression and interactions between microbes and environmental factors, on these interacting partners (see Outstanding Questions). The latter is especially important in the light of the upcoming global challenges, such as global warming and disease control. For instance, the protective effect of Wolbachia against important human diseases in insect vectors [49] is highly dependent upon temperature. Therefore global warming might cause a decrease in protective Wolbachia, undermining ongoing long-term biological control programmes of mosquitoes. In this respect, a broader and more natural approach in studying host-associated microbes is needed, as laboratory studies might often not be directly translatable to the field [41] (Box 3). Although, in this manuscript, our focus is on the traits vector-competence and reproductive manipulation conferred by Wolbachia in arthropod associations, we would like to point out the potential involvement of other host-associated microbes on traits conferred by Wolbachia. As an example, the nutritional symbiosis between Wolbachia and bed bugs showed that Wolbachia–microbe interactions, that is, the complementation of functions by gene exchange between different components of the microbiome, can strongly influence the host phenotype through genetic changes in the symbiont [22,23]. As similar microbe–microbe interactions are not restricted to Wolbachia but also involve other symbionts, a holistic approach should be extended to all symbioses [50].

Finally, the interpretation of data on host–microbe associations has to be done carefully, keeping in mind that small changes in composition and/or abundance of the microbial community might have great phenotypic consequences for the host, as low abundance or rare microbial taxa can represent hub species [51] that are crucial for the host’s functioning, as shown for plants and soil ecosystems [52]. Network analyses of the host-associated microbial communities might represent an important tool [53] for basic insights into interaction dynamics within microbial communities. For instance, this approach has recently revealed that, for several mosquito species (in the laboratory and field), Wolbachia is a highly interconnected taxon, being mostly negatively correlated with other bacteria (i.e., its abundance leads to a reduction in the abundance of other species) [42]. The integration of microbial network analyses with host gene expression networks could provide valuable insights into the complexity of the tripartite interactions.

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