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Diet, Gut Microbes and Host Mate Choice

Understanding the significance of microbiome effects on host mate choice requires a case by case evaluation

*Philip T. Leftwich, Matthew I. Hutchings, and Tracey Chapman**

All organisms live in close association with microbes. However, not all such associations are meaningful in an evolutionary context. Current debate concerns whether hosts and microbes are best described as communities of individuals or as holobionts (selective units of hosts plus their microbes). Recent reports that assortative mating of hosts by diet can be mediated by commensal gut microbes have attracted interest as a potential route to host reproductive isolation (RI). Here, the authors discuss logical problems with this line of argument. The authors briefly review how microbes can affect host mating preferences and evaluate recent findings from fruitflies. Endosymbionts can potentially influence host RI given stable and recurrent co-association of hosts and microbes over evolutionary time. However, observations of co-occurrence of microbes and hosts are ripe for misinterpretation and such associations will rarely represent a meaningful holobiont. A framework in which hosts and their microbes are independent evolutionary units provides the only satisfactory explanation for the observed range of effects and associations.

1. Introduction

Microbes are ubiquitous and can show a range of transient or stable associations with hosts – from commensals and parasites, through to mutualists in which the fate of microbe and host is obligately linked.[1–4] Microbes also exhibit distinct ecological niches within their hosts: commensals may adopt flexible and transient distributions within the gut, whereas obligate mutualists may reside within specialized host structures.[1,3] These factors reflect whether symbionts are intracellular, extracellular, coinherited with their hosts, or acquired from the environment. The development of sequencing technologies has enabled rich descriptions of the communities of non-culturable as well as culturable bacteria that are found on or in different hosts and in novel ecological niches.[1,5,6] One increasing area of interest has been the relationships between hosts and their commensal gut microbes.[7–14]

An example that has attracted considerable interest is the report from *Drosophila melanogaster* fruitflies of a role for gut microbes in determining assortative mating by diet, in which individuals prefer to mate with partners living on, or raised on, the same kind of diet as themselves.[15] Later findings suggested that this effect can be driven by direct effects of gut microbiomes on the mating behavior of their hosts via changes to fly pheromones.[8,9] The evolutionary significance of this mating pattern is that, if sustained, it would have the potential to result in incipient RI of the host. There is much discussion of whether diet-associated mating patterns initiated by microbes could represent a new context for kick-starting RI.[8,10] In addition, this evidence, together with other observations that gut microbes are causal in determining fitness costs in hybrids (e.g., in *Nasonia* jewel wasps[16]), is being employed as strong support for the existence of holobionts (the host plus all its symbiotic microbes)[17] as evolutionary units of selection.[18–21] Proponents of this view argue that host and microbiota should be considered together as a collective whose fitness goals are aligned.[22]

In this article, we discuss the evidence for selection at the level of the holobiont. We begin by discussing the general context of whether associations between microbes and their hosts are always "meaningful," and by outlining why we think true holobionts will be rare. We then cover the various ways in which microbes can alter the mating preferences of their hosts and the recent evidence from *Drosophila*. We conclude by discussing gut microbes and mating preferences in the context of the holobiont theory of evolution. We argue that there is a logical and theoretical disconnect in using evidence that microbes can, in some circumstances, initiate RI of their hosts, in support of the holobiont. We ask why – even if there was an evolutionary association between host and microbe that affected the probability of host RI – would this automatically mean that

Dr. P. T. Leftwich, Prof. M. I. Hutchings, Prof. T. Chapman
School of Biological Sciences
University of East Anglia
Norwich Research Park, Norwich NR4 7TJ, UK
E-mail: tracey.chapman@uea.ac.uk

Dr. P. T. Leftwich
The Pirbright Institute
Ash Road, Pirbright, Surrey GU24 0NF, UK

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the collection of microbes on or in a host is necessarily part of that same evolutionary unit.[23] In addition, species in complex microbiomes have autonomous genomes and may often also exist outside hosts and thus experience highly variable selection pressures. This makes them unlikely to have fitness interests aligned with those of their hosts or of other members of the microbiome, with which they may be in competition. Hence, a more fruitful way in which to view such associations is as communities of individuals whose interests are sometimes aligned and sometimes not.[23,24]

2. Are All Hosts Meaningfully Associated with Bacteria?

Host-associated microbiomes are of interest from many different perspectives, for example, from a desire to understand and characterize the composition of novel microbiomes, to manipulate microbes in order to study effects on host health and behavior or to understand the evolutionary interplay between hosts and their microbes. Comprehensive sequencing of 16S rRNA genes derived from plants and animals have aided these efforts and have demonstrated the ubiquity of microbiome-host associations.[3] It is clear that microbes often have important effects on host biology.[1–3,25] This has fuelled a concerted effort to determine whether individual plant or animal species play host to recognizable microbes or microbial communities. In particular, there has been a drive to determine to what extent each host has a "core microbiome" of particular microbial species. Recurrent associations of particular bacterial species with a specific host are often used as a proxy for answering this question. However, recent work emphasises that core microbiomes could also exist at the functional level, and comprise interchangeable functionally redundant species.[26]

It is well known that microbes can colonize hosts and exert significant influences on host fitness and biology without the requirement for an evolutionary relationship.[27] In addition, if the host is not the usual habitat for the microbe, repeated acquisition of bacteria from the environment may in fact represent a microbial evolutionary dead-end.[6] Many hosts may exist in an environment where diets are limiting, with important components supplied by microbes with which they form varying degrees of associations.[1,3,27] However, many benefits to hosts may arise as by-products of bacterial metabolism. Hence, the host might gain but not because these products evolved to benefit the host.[23]

The ubiquity of microbes means that most analyses of hosts are likely to identify a collective of host-associated bacteria, which could be labeled a "microbiome." However, this should not lead to the assumption that all or even some of these bacteria are of vital importance to the host. Though not always explicit, there is a tendency to assume that co-occurrence indicates the existence of a functional or adaptive explanation for either or both parties. We argue that the starting point should always be that co-occurrence has no special significance unless there is evidence to the contrary. For example, the presence of microbes in the gut that possess the ability to utilize specific nutrients, is some distance from support for the idea that they do this to pass nutritional benefits the host. For instance, bacteria (*Klebsiella* spp) from the enteric group of gamma proteobacteria, occur in the gut of the Tephritid fruitfly *Ceratitis capitata*. These bacteria can fix nitrogen in situ in the medfly gut.[28,29] There has been increasing interest in the potential importance of this source of N_2 fixation to the host[30] to compensate for nitrogen-poor host diets. This has been used as a rationale for probiotic supplementation.[31,32] However, the fate of fixed N_2 – whether it becomes directly incorporated in host tissues and whether any host benefit is specifically associated with N_2 fixation by *Klebsiella* – is not yet known. Tracking techniques confirming that bacterial-derived N_2 can be incorporated within host biomass[33,34] (as occurs in termites,[35] shipworms,[33,36] and various marine invertebrates[37]) are required.

Another example is found in *Aedes* and *Anopheles* mosquitoes. Bacteria ingested by developing mosquito larvae are reported to be essential for developmental viability.[38] However, in a recent study in *Aedes aegypti* and *Anopheles gambiae* it was shown that the ingestion of living (but not dead) eukaryotic or prokaryotic cells of diverse types caused the same fitness benefits to the hosts.[39] This suggests that the ingestion of any living microbes can provide the host benefit. Thus, the significance of co-occurrence needs to be interpreted with great caution. To assess whether the co-occurrence is indicative of a potential evolutionary relationship, it is necessary to satisfy the following criteria: i) Is there a mechanism by which recurrent exposure can occur; ii) is there fidelity of recurrent exposure; iii) is there any dependence of one or both parties on the other; iv) what are the ultimate fitness effects for hosts and microbes of the presence and absence of each party.

3. Hosts and Their Microbes as Holobionts

The ultimate extrapolation of tight co-occurrence between host and microbes is to view them as an evolutionary individual, or holobiont. However, for such an entity to behave as an evolutionary individual, the constituent parts need to show a coincidence of fitness interests and a mechanism for co-inheritance[40,41] as any other scenario will be unstable. The mechanisms by which this can be achieved include cotransmission/coinheritance between host and microbes, mutual fitness benefits and repression of competition among all interacting parties (**Figure 1**). Not all of these criteria have to be realized perfectly in order for this to occur and selfishness can persist even under tight co-dependency.[42] Hence, even the tightest of mutualisms is expected to exhibit some level of conflict with the host,[43] potentially leading to arms races or red queen type dynamics.[6] Policing by hosts to eliminate symbionts expressing selfish behavior is also possible[44] though no examples from insects are yet reported.

If sufficient criteria are met, a non-fraternal ("egalitarian") association comprising non-related species can result.[40] However, as the number of interactants increases (e.g., as more gut microbial species are involved) the probability of ensuring efficient co-transmission and policing of selfishness among all parties involved will decrease. This is the primary reason for why the concept of a holobiont as an evolutionary individual comprising hosts plus many microbes (e.g., the gut microbiome) is probably a stretch, at least most of the time. If the association is between the host and a small portion of the microbiome, then the concept of the bona fide holobiont is further diluted. This also explains why, when evolutionary associations between hosts and microbes do occur (e.g., in the obligate symbiont *Buchnera*

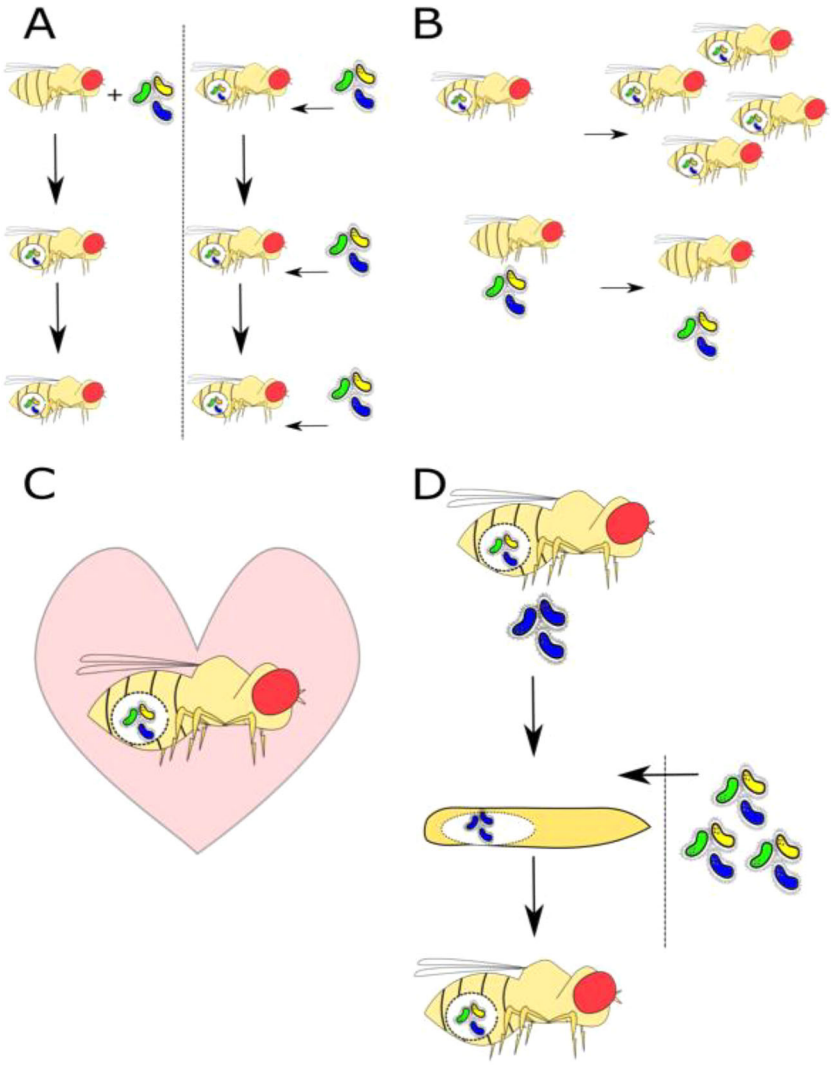

Figure 1. Four key questions for the individual as a potential holobiont. a) How is co-transmission/coinheritance ensured between host and microbes? A holobiont must ensure high fidelity and recurrent transmission of host plus microbes (panel A, left hand side), or recurrent environmental acquisition (panel A, right hand side), through evolutionary time. b) Are there identifiable, specific, fitness benefits for both parties as a result of the association? There must be specific and identifiable mutual fitness benefits arising from the coassociation of host and bacteria, such as increased number of descendants. c) How is repression of competition ensured amongst all interacting parties? In a holobiont, the evolutionary interests of all interacting parties are aligned and mechanisms to achieve that may be required. d) Is all of the microbiome part of the holobiont? For a holobiont to represent a single evolutionary unit, all parties should have coincident fitness interests, hence show a unitary response to selection. This is unlikely to occur if some of the microbiome is inherited through the host life cycle and some is flexibly attained from the environment, because some portion of microbe fitness is then host-independent.

aphidicola and its *Acyrthosiphon pisum* pea aphid host[1] they tend to involve a host and a single symbiont or hosts transitioning between an old a newer symbiont.[45] Symbiont replacement appears to be rare,[46] but some groups such as scale insects, psyillids, whiteflies, and mealybugs retain relics and evidence of multiple gains and losses.[6]

Microbes do not have to be maternally transmitted in order to have significant effects on their hosts.[5,27,47] What is of greater importance is that exposure of host to mutualistic symbiont occurs with high fidelity. For example, in the association between the bobtail squid and its bioluminescent symbiotic bacterium *Vibrio fischeri*, the symbiont is acquired in a recurrent and efficient fashion from the environment.[48] Species in which there is recurrent environmental transmission of microbes can sometimes express a limited amount of plasticity in the symbionts with which they associate, for example, as in some leaf cutter ants.[49] Though this introduces risks into the intergenerational transmission of symbionts, it may also allow hosts to show adaptive symbiotic associations, for example, with symbionts that are adapted to the prevailing environment.[50]

The most fruitful way forward is to realize that co-occurrence can be observed for many different proximate or ultimate reasons. Microbes may represent transient passengers, long term fellow travellers, parasites or indispensable companions, and co-occurrence per se cannot necessarily determine which of these is correct.[23] Microbes of these varied types may have no,

minimal or significant effects on hosts, whether they form an evolutionary association or not.

4. How Endosymbionts Can Affect the Mating Patterns of Their Hosts

In this section we briefly review the ways in which microbes can alter the mating patterns of their hosts, with the potential to lead to RI, and why they might do so. We summarize briefly the empirical evidence and consider whether such effects could also occur in associations between commensal gut bacteria and their hosts, with a particular focus on the relationships affecting diet and mate choice.

4.1. Heritable Symbionts Develop Incompatibilities with Host or Symbiont Alleles and Direct Host Mating Behavior

Heritable symbionts can potentially influence the reproductive behavior of their hosts if they develop genetic incompatibilities with host alleles.[6] These are analogous to incompatibilities that can occur between mitochondrial genotypes and nuclear genes, or between symbionts themselves, and both can drive host RI[51] (**Figure 2**).

Genetic incompatibilities with host alleles can drive RI if gut symbionts are maternally transmitted and come to influence mate choice, because host females are selected to discriminate against males with nuclear genotypes that are incompatible with the females' symbiont type. Here, the effect of the microbes on host mating behavior is indirect. Such incompatibilities are predicted to show rapid evolution, as hosts show counter evolution to respond to changes in their symbionts that arise due to the selfish behavior of the symbionts or because of genetic drift.[52] These changes are expected to accelerate post zygotic RI between populations or, potentially, even within them.[53]

The best-known examples of symbiont-host incompatibilities are found in individuals harbouring *Wolbachia*, an intracellular bacterial symbiont that occurs in many invertebrate species[2,54,55] and which is vertically transmitted through the maternal line, linking the evolutionary fates of host and bacteria over time. The many effects of *Wolbachia* on its hosts include cytoplasmic incompatibility (CI), feminization, male killing and the induction of parthenogenesis.[2,54–57] These effects benefit the *Wolbachia* as they promote its spread via maternal transmission through the elimination, in various ways, of male hosts from the population. *Wolbachia*-induced uni-directional CI is a particular focus for understanding host biology because, in theory, CI can lead to host RI,[58–60] because CI increases the fitness of infected females relative to uninfected females, and hence may select *Wolbachia*-derived mechanisms for avoiding mating with uninfected females.

It is generally thought that unidirectional CI is not a strong driver of host RI due to variable *Wolbachia* infection between different populations and gene flow.[61] Hence, the evolution of host RI under these conditions requires additional mechanisms. Several authors have asked whether there are additional effects of *Wolbachia* infection on host mate choice outside CI that could promote *Wolbachia*

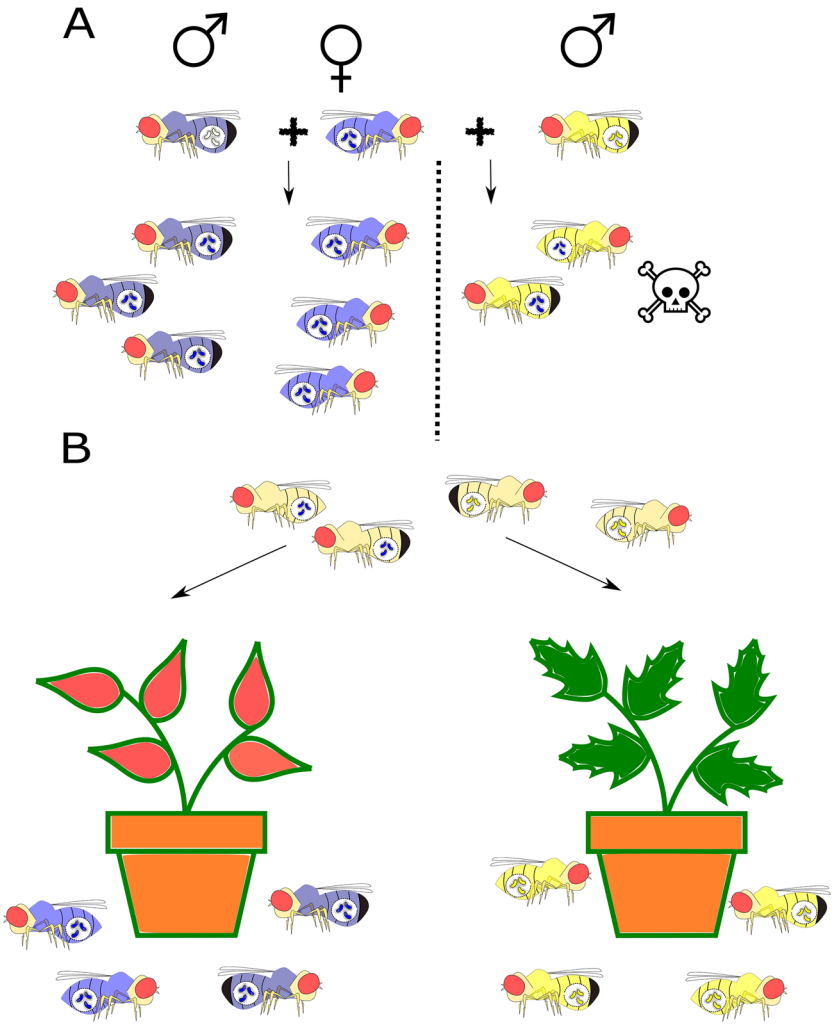

Figure 2. How microbes can select on the mating behaviour of their hosts. a) Heritable symbionts develop incompatibilities with host alleles and direct host mating behaviour. Here, there are compatibilities between host nuclear and symbiont genomes (indicated by matching colours). Symbionts are maternally transmitted, hence, symbionts can drive the benefits of choice for matings with males that have a nuclear genome that is compatible with the female's symbiont (left hand side). The consequence of a mismatched mating (right hand side) is that offspring have a nuclear genome that is mismatched with their maternally inherited symbionts. Hence such matings are selected against by the symbiont. Similar incompatibilities occur, and can drive the evolution of host mating, in bi-directional incompatibilities between hosts harbouring different *Wolbachia* strains (see text). b) Microbes affect host food preferences directly. Here, the symbiont itself changes habitat (or food) preference, causing the hosts to become located in different places. This has the potential to eventually result in reproductive isolation in allopatry (blue versus yellow host genotypes), given sufficient contribution of genetic drift, likely aided by selective processes, driven by fitness benefits and costs to either party on the new hosts.

spread, for example, through direct manipulation of host attractiveness and mating preferences.[12] For example, *Wolbachia* infection can increase female body size, potentially through an interaction with insulin signalling.[62] Hence, if larger females are more attractive, this could increase *Wolbachia* spread. Though *Wolbachia* infected females are also predicted to be more fecund and effects of infection on male mating activity and mate choice are reported,[63] these effects are by no means consistent.[12,59,64] This suggests that our understanding of the ability of symbionts to manipulate the mating behavior of their hosts, both under scenarios where such effects are predicted or not, remains far from complete. In contrast to uni-directional CI, bidirectional CI, in which neighboring populations are infected with different *Wolbachia* strains, can more easily facilitate host RI[65,66] because of bidirectional mating incompatibilities. At first, it was thought that the conditions for stable co-infection could not exist. However, recent theory counters this view[66] and suggests that further research into such incompatibilities would be useful, including for insect control.[67]

The principles of these systems theoretically extend beyond *Wolbachia* to any heritable symbionts that display sufficient fidelity to their host. Over time these could develop incompatibilities with other symbiont alleles and thus influence host mating behaviour. Elevated expression of selfishness and conflict within a host-symbiont association, is expected to increase the chance of rapid divergence. It is also possible, given sufficient stability and co-transmission of host and microbe, that associations with non-heritable, environmentally-acquired symbionts could also show such incompatibilities and influence host mate choice. Whether such incompatibilities exist or result in elevated levels of speciation, within symbionts based in the gut rather than via intracellular symbionts such as *Wolbachia*, is not yet clear.

4.2. Microbes Affect Host Food Preferences Directly

Another possibility is that the symbiont itself changes habitat (or food) preference, causing the hosts to be located in different places and to become reproductively isolated in allopatry[68,69] (Figure 2). Examples of this phenomenon are known in the mutualistic heritable symbionts of insects, such as symbionts affecting host plant use or tolerance to heat.[5] For example, there are distinct host races of the pea aphid (*Acyrthosiphon pisum*) that feed on different host plants,[70] and co-association between the symbiont *Regiella insecticola* and the red clover host have been reported. Some aphid genotypes associated with this symbiont have higher fitness when feeding on clover.[71] This, along with the reported effect of the symbiont on host dispersal and the timing of sexual morph production, could potentially reduce gene flow between hosts living on different plants and promote the host plant specialization[72] by selecting for *R. insecticola*-infected aphids with a preference for clover. However, facultative symbionts may also compete with their hosts for nutrition, meaning that in some cases symbionts do not supplement poor host diets but instead make them even less available to hosts.[5]

What is implicit in the arguments detailed above in sections 4.1 and 4.2 is that evolution in the host is driven by symbiotic microbes, to avoid incompatibilities, or that microbe fitness and transmission is enhanced through the evolution of mate choice in the host. Here, changes to host mating preferences are driven by the symbionts and there can be a range of different effects on host fitness. What is also important to note in the context of the discussion in the next section, is that the relationships leading to host RI usually involve one host and one microbe.

4.3. Microbes Affect Host Mate Choice Directly

Experiments on gut microbiomes in fruitflies appear to imply that it is the food supply, and the bacteria on it, that directly initiates a change in host mate choice.[8,9] This would represent an immediate and reversible phenomenon caused directly by the presence or absence of bacteria. In principle, individuals feeding on different diets could pick up novel microbiomes very quickly and, if those interactions somehow eventually increased the probability of allopatry as shown in Figure 2, this could, along with other selective processes, contribute to RI. The key point is that to detect the chain of causality in this process requires manipulative approaches in addition to correlational patterns. However, shifts of hosts to new diets is not without risk, and for hosts with well-established symbionts it may increase the risk of symbiont loss. By the same reasoning, expansion of hosts into new habitats with differing abiotic conditions may also present risk to host-symbiont associations, particularly since symbionts often appear to be much more heat sensitive than their hosts.[6] The diversity of studies showing evidence of the ways in which microbes can alter the breeding patterns of their hosts, potentially leading to host RI, all rely on an understanding of the nature of the association and the fitness costs and benefits involved for both parties. In the next section we discuss these same themes further in the context of the potential role of the gut microbiome in diet-associated mate choice in *Drosophila*.

5. A Role for Gut Microbiome Symbionts in Driving Host RI in *Drosophila*?

Reports in the fruitfly *Drosophila melanogaster* have asserted that assortative mating by diet, in which individuals prefer to mate with partners living or raised on the same kind of diet as themselves,[15] can be driven by direct effects of gut microbiomes on the mating behavior of their hosts. The evidence for such effects has been derived from experiments in which the mating preferences of flies have been tested when i) maintained on different diets; ii) cured of their microbiomes with antibiotics; and (iii) re-inoculated with specific bacterial species.[8,9] These experiments showed evidence that significant assortative mating by diet was eliminated by antibiotic treatment and then restored by adding back the gut bacterial species *Lactobacillus plantarum*. A remarkable and puzzling observation was that the effect of diet on assortative mating, and its prevention by antibiotic treatment, was effectively instantaneous, indicating a direct effect of the presence of the bacteria. However, other studies showed that neither the significant positive assortative mating by diet, nor the involvement of gut bacteria in this process, is consistent within or across different *Drosophila* strains either instantaneously or across generations.[7,11,13,14,15,73]

Nevertheless, the discussion of gut microbiomes in *Drosophila* in the light of this original experiment has been used as support for a role of bacteria in RI.[21] This is a surprising interpretation because the gut microbes of *D. melanogaster* are reported in these studies to be relatively flexible and acquired from the diet.[74,75] Hence, the composition of the gut community appears to depend upon the specific diet ingested at any particular moment, which varies across an individual's lifetime[76] and may also be different for each sex.[77] There is little evidence for vertical transmission of gut-associated bacteria in this species, nor any evidence for recurrent exposure to parental gut bacteria in each generation. It is possible that there is host sorting/screening of microbiota from the total possible set of microbes to which the host is exposed, for example, to let the "right ones in."[78–80] However, this seems incompatible with the observed apparent flexibility in the microbiome of *D. melanogaster* according to the prevailing diet[74,75] or environmental conditions[81] unless it can be demonstrated that the host is actively or passively screening for a different form of core microbiome, based on functions rather than specific species.[26]

For there to be an evolutionary association between the *D. melanogaster* host and gut microbes that has the potential to initiate RI, we need to consider whether there are other ways in which co-inheritance, stability, or recurrence of the association between host and environmentally-acquired bacteria through evolutionary time could occur.[24] Mechanisms that could favor stable and potentially evolutionary co-associations are: i) the degree of host food specialization (assuming that communities of bacteria are stable for any given diet type); ii) social interactions of the host (the probability of contacts between individuals from the same diet types rather than between different ones); and iii) the interplay of the microbiota with the host immune system (e.g., via specialized structures such as the bacteriocytes of pea aphids[1,3] or the gut physiology of Tephritids such as the olive fruit fly[82]) such that only a restricted and characteristic set of microbes of the total available ends up forming the microbiome. Food specialization could determine the probability with which gut microbes are reacquired each generation.[74] In addition, food specialization could also interact with cuticular hydrocarbon profiles that mediate host attractiveness (e.g., in *Rhagoletis* Tephritid fruit flies[83]). The degree to which gut microbial communities vary through an individual's lifetime are also important as recurrent contact across generations could be reduced if microbe composition varies widely throughout the different host life history stages. The *Drosophila* story has taken a recent interesting twist, with a recent report of a stable association between *D. melanogaster* and a gut microbiome bacterium *Acetobacter thailandicus* isolated from wild flies derived from figs[84] (Box 1). The new study is interesting, yet the case for a previously undescribed mutualism in this species is not yet fully supported. Hence further investigations of this potentially significant relationship should prove insightful.

Key to consider is to identify whether hosts, microbes or both stand to gain by the altered mating patterns and thus whether any such associations between them have any evolutionary significance (Box 2). For example, it is hard to envisage how bacterially-induced mating assortment by diet could be strongly naturally selected over time, unless it somehow resulted in increased transmission of the bacteria that caused the assortative mating to the descendants of those mating pairs. Again, such effects are not yet known. From the host's perspective, it is possible that the promotion by gut bacteria of positive assortative mating by diet might be beneficial in the context of bringing together individuals to mate that show local dietary adaptation. However, there is as yet little evidence for recurrent exposure or microbiome stability in *D. melanogaster*, so no evidence that the current composition of the gut microbiome is indicative of the

BOX 1
A new Drosophila – gut microbe mutualism?

Pais et al.[84] reported recently that a gut microbe found in wild *D. melanogaster*, *A. thailandicus*, shows greater persistence in the *D. melanogaster* gut than for other species of gut microbes tested. Furthermore, the bacteria conferred some benefits to host offspring, and benefits to the bacterium were also reported as increased transmission via the fly gut. Pais et al.[84] propose that the *D. melanogaster/A. thailandicus* co-association represents a previously undescribed mutualism. However, some key questions remain. The stability assay used to establish persistence of bacterial species in the gut was focused on adults and may not have included all potential windows for colonization and establishment. A full range of colonization routes is is likely to be required in order to fully determine which bacteria colonize and which are merely present in the environment. Benefits to hosts were also seen for other bacteria and host benefits in laboratory flies appeared similar following infection by either the persistent *A. thialandicus* or another non-persistent *Acetobacter* strain (OTU2753), rendering no extra benefit to any potential mutualism, though of course the natural context might be different. The benefits to *A. thialandicus* of associating with the *D. melanogaster* host were reported to be increased bacterial transmission. In the absence of knowledge about the portion of A. thialandicus fitness that is realised inside and outside the *Drosophila* host, it is not yet known how much of a fitness benefit this might represent. The relative proliferative rates of gut microbes inside and outside the host will be key to interpreting the full spectrum of potential fitness effects in this context. The recent study employed culture-dependent techniques and will necessarily have focused on abundant, readily culturable bacteria. It does not as yet represent a full survey and will have been focused on abundant bacteria, or those that grow abundantly within the host/and or culture medium. These interesting recent results support the idea that the microbial species associated with the gut of *D. melanogaster* vary in their degree of co-association but do not yet appear to fully support a new mutualistic relationship.

BOX 2
Principles for evaluating the proximate/ultimate significance of effects of acquired gut microbiomes on host mating preferences.

Consistency and Persistence of Assortative Mating by Diet. It is important to understand how diet affects mating preferences, and in particular whether any such effects reflect transient or evolved phenomena. Related to this is the consistency with which such effects are observed. For example, significant positive assortative mating by diet in *D. melanogaster* seems variable across strains of *Drosophila*.[7–9,11,13,14] The reasons for this variability are not yet understood. It is also key to establish whether any assortative mating that does occur is stably linked to the presence/absence or composition of specific bacterial species in the gut. If this is only loosely the case, or is a transient phenomenon, then, although it is important to understand the mechanisms involved, it suggests that specific gut bacteria are unlikely to have a general role in driving the evolution of host mating preferences.

Food Specialism versus Generalism. This is important in order to understand the likelihood of ecological adaptation as well as recurrent exposure to the communities of bacteria that live on/in different diets. For example, *D. melanogaster* appears to exhibit a generalist lifestyle with respect to diet,[90,91] with food use varying across the lifetime[76] and in each sex.[77] This would seem to provide only weak support for the idea that the food preferences of this species would reinforce recurrent exposure to the same potential pool of gut bacteria over time. This is expected to decrease the potential for evolutionary coassociations between hosts and gut microbes in this species.

Flexibility of Gut Microbiome Community Within and Across Generations and Populations. Variation in the composition of the gut microbiome will impact on the potential for evolutionary co associations and hence how hosts and their microbiomes respond to selection. With high variability in the gut microbe community across time, space and sex, the potential for strong evolutionary co-associations is again reduced.

Mechanism of Recruitment of Acquired Gut Bacteria into Hosts in Each Generation. Should a role for gut microbiomes in long term host mating preferences be supported, then it is important to understand how coinheritance, or recurrent exposure of hosts to the same communities of gut bacteria, occur each generation. We note that the mechanisms by which vertical transmission can occur among hosts and acquired extracellular symbionts, such as the gut bacteria, are relatively understudied.[16,92,93]

Fitness Benefits of Assortative Mating by Hosts. Of central importance is to understand the full fitness consequences for hosts and gut microbiomes of their associations over the short and long term. In order to understand the significance of the effects of each party on the other, and specifically whether these effects have any longer-term significance, we need to know whether hosts or microbiomes or both benefit from the expression of assortative mating and if so how and over what timescale.

dietary adaptation status of the host. Hence, there seems limited opportunity for the host to benefit from gut bacteria skewing their host mating preferences or causing host divergence. Therefore, it is not clear that this assortative mating has any longer term, ultimate significance. Any potential short-term benefits of the assortative mating are also unclear and have not yet been identified in these scenarios.

6. Hosts, Gut Microbiomes and the Holobiont

Studies of the effects of gut microbiomes in *D. melanogaster* fruitflies, and in *Nasonia* jewel wasps have been suggested to support the holobiont idea.[10,16] The *Drosophila* studies in particular have fuelled increasing interest in the possibility that selection can act on the holobiont in a way that can lead to host speciation.[20] Increasing interest in microbiomes and their effects on hosts is welcome, both for acquired versus inherited symbionts. However, several important qualifications are needed in order to correctly evaluate the current claims concerning holobionts and RI.[4,24,85]

D. melanogaster plus its gut bacteria seem unlikely to represent a unitary "holobiont," but instead, as with many such associations, a set of shifting alliances of fitness interests between host and microbes. *D. melanogaster* is also not completely dependent upon its gut microbes, because axenic flies can be created via egg dechorionation and are viable, though slower to develop, but can show normal adult female fecundity.[27] It is logical to assume that, even if the same functional types of bacteria typically form the gut bacterial community in this host, this does not result from co-inheritance or recurrent exposure between gut microbes and hosts.[4] Therefore, gut bacteria and their fruit fly hosts have little shared interest in each other's long term futures. Difficulties with the application of the holobiont idea to this example arise when taking into account the reported flexibility in the microbiome of *D. melanogaster* according to the prevailing diet.[74,75] The host does not appear to screen out all but a set of core microbes.[78–80] This does not preclude an evolved response to certain bacterial species – recurrent but not fixed interactions with certain species could be predicted to produce such a response, and research in *Drosophila* has shown that specific genes are differentially

expressed in the presence/absence of bacteria in the fruit fly gut.[25] However, no significant associations are found between the host transcriptome and the species composition of the gut microbiome, indicating that although the host reacts strongly to the general presence of bacteria, it does not do so in a taxon-specific manner. This coupled with the variable phenotypic responses to bacteria reported above, would indicate that there is little evidence for evolved relationships with specific species of bacteria in *Drosophila*, where we would instead expect fidelity of the observed responses. Hence, a general role for gut microbiomes in driving positive assortative mating by diet in *D. melanogaster* is not supported.[13]

Complex microbiomes, comprising multiple species, such as those found in *D. melanogaster* and most invertebrates to-date, lack mechanisms to ensure fidelity and are only loosely associated with hosts. In fact, many species that make up microbiomes can live perfectly well outside the host. This begs the question of whether this part of their environment is excluded from the "holobiont?" The environment outside the host presumably exerts very different selective forces – this would fall outside the purview of a holobiont and exclude consideration of potentially key parts of microbial life histories. Complex microbiomes are highly unlikely to be transmitted vertically in an intact manner – hence individual constituent species will gain through increasing their own transmission. The number of different species present in a microbiome makes collective co-operation even more unlikely and constituent members are likely to often compete with each other. Hence a microbiome seems much better viewed as a community in which there is conflict as well as co-operation.

The finding that associations between hosts and their gut microbes may have some features consistent with a holobiont, does not mean that this is necessarily the sole explanation for their association, as other alternatives are usually also possible. To take another example, the loss of fitness in hybrids between *Nasonia* jewel wasps appears to be associated with their gut microbiota.[16] The finding is that in hybrids, the growth of the gut microbiota appears to become unregulated, contributing to hybrid fitness loss and potentially to mechanisms to reduce the probability of hybrid matings. However, as outlined in refs. [24,86], there are other, more parsimonious possibilities such as increased growth of bacteria in the guts of unhealthy hybrids, potentially also promoted by disrupted immune systems.

The relationships between hosts and their symbiotic microbes can be viewed in an analogous manner to interactions between any other interacting parties across the biological hierarchy (e.g., between different sexes,[87] social castes[41] or members of an interacting community.[24] When the fitness interests of the interacting parties coincide, they are more likely to cooperate and respond similarly to selection. However, whenever those interests do not coincide, each party will be selected to pursue a strategy that enhances their own fitness, potentially even with some cost to the other.[87] Hence, the type of association between interactants can modulate the expected mutual effects of each party. More importantly, an understanding of the nature of the relationship between symbiont and host is essential for interpreting what the effects of microbes on hosts might "mean" in an ultimate sense.[4,24,86]

Hosts may provide novel ecosystem niches, for example, for vital functions that they require from bacteria and these may be occupied by any of a number of different environmentally-acquired bacteria. This is analogous to the different ways in which ecosystems themselves may be characterized, for example, in terms of species level versus functional diversity. It may be that consideration of functional, rather than species level diversity, can provide additional explanatory power for microbiome composition. Such an approach emphasizes the redundancy of individual bacterial species that can be substituted for others that can occupy the same niche. This does not have to be an active process by the host, which could simply provide a resource for suitable bacteria. From the microbial perspective, some bacteria will attempt to exploit these niches, while others may attempt to grow and utilize resources which would harm their host. Hence improvements in our ability to identify niches and characterize microbiomes based on function as well as by species may be particularly fruitful.

7. Conclusion

The growing interest in the effects of symbionts, and in particular of acquired gut bacteria, on the mating behavior of their hosts is stimulating much discussion into the significance of the resulting outcomes for driving divergence in host reproductive traits.[4,24,86] It is also contributing to a broader consideration of the extent to which the host and its microbiome can act as a selectable "individual" or holobiont.[88] Key to understanding the strength of selection at this level is to understand the fitness effects of any manipulations by microbes of their hosts and specifically which parties benefit. Along with this, the way in which microbes are inherited or re-exposed to their hosts in each generation is also key in ascertaining the relative potential for selection at the level of the holobiont. In general, for commensal gut bacteria and hosts we expect selection at the level of the holobiont to be weak due to the lack of mechanisms for co-inheritance and hence shared interest in each other's futures.[4,13,24] This contrasts with the well-established theory and empirical data on the manipulation of host mating behavior of intracellular and vertically transmitted symbionts, such as *Wolbachia*.[64]

What is the significance, then, of instances in which apparently commensal microbiomes affect the mating behavior and preferences of their hosts? Such effects appear inconsistent and variable. Hence, the possibility that such symbionts drive host divergence directly through alterations to mating preferences is low because differential modes of transmission will rapidly decouple symbionts from hosts. A valuable approach will be is to gain a better understanding of the fitness benefits and costs of such effects for hosts and microbes and to view their relationship as members of a community of interacting individuals in which there is both conflict and co-operation. This is likely to be more fruitful than a focus on selection at the level of the holobiont, as this is only possible under conditions in which the evolutionary interests of hosts and the specific microbe or microbes involved are aligned. Such scenarios are susceptible to cheating, particularly when microbiomes are

complex, and the conditions under which such higher level selection will occur are expected to be rare.[23,41,89]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

gut microbiome, holobiont, reproductive isolation, selection, speciation, Symbiosis, unit of selection

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[1] A. E. Douglas, *Ann. Rev. Entomol*. **1998**, *43*, 17.
[2] J. H. Werren, L. Baldo, M. E. Clark, *Nat. Rev. Microbiol*. **2008**, *6*, 741.
[3] N. A. Moran, J. P. McCutcheon, A. Nakabachi, *Ann. Rev. Genet*. **2008**, *42*, 165.
[4] N. A. Moran, D. B. Sloan, *PLoS Biol*. **2015**, *13*, e1002311.
[5] K. M. Oliver, P. H. Degnan, G. R. Burke, *N. A. Moran. Ann. Rev. Entomol*. **2010**, *55*, 247.
[6] G. M. Bennett, *N.A. Moran. Proc. Natl. Acad. Sci. USA* **2015**, *112*, 10169.
[7] S. Pavković-Lučić, *Arch. Biol. Sci*. **2009**, *61*, 105.
[8] G. Sharon, D. Segal, J. M. Ringo, A. Hefetz, J. Zilber-Rosenberg, E. Rosenberg, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 20051.
[9] G. Sharon, D. Segal, J. M. Ringo, A. Hefetz, J. Zilber-Rosenberg, E. Rosenberg, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 4853.
[10] G. Sharon, D. Segal, J. Zilber-Rosenberg, E. Rosenberg, *Gut Microbes* **2011**, *2*, 190.
[11] M. A. Najarro, M. Sumethasorn, A. Lamoureux, T. L. Turner, *Peer J*. **2015**, *3*, e1173.
[12] D. Arbuthnott, T. C. Levin, D. E. L. Promislow. *J. Evol. Biol*. **2016**, *29*, 461.
[13] P. T. Leftwich, N. V. E. Clarke, M. I. Hutchings, T. Chapman, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E2154.
[14] P. T. Leftwich, N. V. E. Clarke, M. I. Hutchings, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 12767.
[15] G. Kilias, S. N. Alahiotis, M. Pelecanos, *Evolution* **1980**, *34*, 730.
[16] R. M. Brucker, S. R. Bordenstein, *Science* **2013**, *341*, 667.
[17] L. Margulis, R. Fester (Eds.), *Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis*, MIT Press, Cambridge, MA, USA **1991**, pp. 1–14.
[18] E. Rosenberg, O. Koren, L. Reshef, R. Efrony, I. Zilber-Rosenberg, *Nat. Rev. Microbiol*. **2007**, *5*, 355.
[19] I. Zilber-Rosenberg, E. Rosenberg, *FEMS Microbiol. Rev*. **2008**, *32*, 723.
[20] R. M. Brucker, S. R. Bordenstein, *Trends Ecol. Evol*. **2012**, *27*, 4430451.
[21] E. Rosenberg, I. Zilber-Rosenberg, *Microbiome* **2018**, *6*, 78.
[22] S. R. Bordenstein, K. R. Theis, *PLoS Biol*. **2015**, *13*, e1002226.
[23] D. C. Queller, J. E. Strassmann, *Biol. Philos*. **2016**, *31*, 855.
[24] A. E. Douglas, *J. H. Werren. MBio*. **2016**, *7*, e02099.
[25] A. J. Dobson, J. M. Chaston, P. D. Newell, L. Donahue, S. L. Hermann, D. R. Sannino, S. Westmiller, A. C. N. Wong, A. G. Clark, B. P. Lazzaro, A. E. Douglas, *Nat. Commun*. **2015**, *6*, 6312.
[26] A. Bost, V. G. Martinson, S. Franzenburg, K. L. Adair, A. Albasi, M. T. Wells, A. E. Douglas, *Mol. Ecol*. **2018**, *27*, 2834.
[27] E. V. Ridley, A. C. N. Wong, S. Westmiller, A. E. Douglas, *PLoS One* **2012**, *7*, E36765.
[28] K. M. Murphy, D. S. Teakle, I. C. Macrae, *Appl. Environ. Microbiol*. **1994**, *60*, 2508.
[29] A. Behar, B. Yuval, E. Jurkevitch, *Mol. Ecol*. **2005**, *14*, 2637.
[30] B. Yuval, E. Ben-Ami, A. Behar, M. Ben-Yosef, E. J. Jukevitch, *J Appl. Entomol*. **2010**, *137*, 39.
[31] S. Gavriel, E. J. Jurkevitch, Y. Gazit, B. Yuval, *Appl. Entomol*. **2011**, *135*, 564.
[32] H. Hamden, M. M. Guerfali, S. Fadhl, M. Saidi, C. Chevrier, *J. Econ. Entomol*. **2013**, *106*, 641.
[33] C. P. Lechene, Y. Luyten, G. McMahon, D. L. Distel, *Science* **2007**, *317*, 1563.
[34] A. A. Pinto-Tomás, M. A. Anderson, G. Suen, D. M Stevenson, F. S. T. Chu, F. W. W. Cleland, P. J. Weimer, C. R. Currie, *Science* **2009**, *326*, 1120.
[35] J. R. Benemann, *Science* **1973**, *181*, 164.
[36] Y. A. Luyten, J. R. Thompson, W. Morrill, M. F. Polz, D. L. Distel, *Appl. Environ. Microbiol*. **2006**, *72*, 412.
[37] C. L. Fiore, J. K. Jarett, N. D. Olson, M. P. Lesser, *Trends Microbiol*. **2010**, *18*, 455.
[38] K. L. Coon, K. J. Vogel, M. R. Brown, M. R. Strand, *Mol. Ecol*. **2014**, *23*, 2727.
[39] L. Valzania, V. G. Martinson, R. E. Harrison, B. M. Boyd, K. L. Coon, M. R. Brown, M. R. Strand, *PLoS Negl. Trop. Dis*. **2018**, *12*, e0006638.
[40] D. C. Queller, *Phil. Trans. Roy. Soc. B* **2000**, *355*, 1647.
[41] A. F. G. Bourke, *Principles of Social Evolution*. Oxford University Press, New York **2011**.
[42] S. A. Frank, *Am. Nat*. **1997**, *150*, S80.
[43] R. A. Chong, N. A. Moran, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 13114.
[44] S. A. Frank, *Evolution* **2003**, *57*, 693.
[45] A. Lamelas, M. J. Gosalbes, A. Manzano-Marín, J. Peretó, A. Moya, A. Latorre, *PLoS Genet*. **2011**, *7*, e1002357.
[46] R. A. Chong, N. A. Moran, *ISME J*. **2018**, *2*, 898.
[47] E. G. Ruby, M. Urbanowski, J. Campbell, A. Dunn, M. Faini, R. Gunsalus, P. Lostroh, C. Lupp, J. McCann, D. Millikan, A. Schaefer, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 3004.
[48] L. K. Lee, E. G. Ruby, *Appl. Environ. Microbiol*. **1994**, *60*, 1565.
[49] S. B. Andersen, S. H. Yek, D. R. Nash, J. J. Boomsma, *BMC Evol. Biol*. **2015**, *15*, 2.
[50] N. A. Moran, *MBio*. **2016**, *7*, e01904.
[51] R. S. Burton, R. J. Pereira, F. S. Barreto, *Ann. Rev. Ecol. Evol. Systemat*. **2013**, *44*, 281.
[52] J. H. Werren, S. Richards, C. A. Desjardins, O. Niehuis, J. Gadau, J. K. Colbourne, Nasonia Genome Working Group, *Science* **2010**, *327*, 343.
[53] R. B. Corbett-Detig, J. Zhou, A. G. Clark, D. L. Hartl, J. F. Ayroles, *Nature* **2013**, *504*, 135.
[54] A. A. Hoffmann, M. Turelli, *In Fluential Passengers*, (Eds: O'Neill S. L., Hoffmann A. A., Werren J. H.), Oxford Univ. Press, **1997**, pp. 42.
[55] J. H. Werren, *Ann. Rev. Entomol*. **1997**, *42*, 587.
[56] W. J. Miller, L. Ehrman, D. Schneider, *PLoS Path*. **2010**, *6*, e1001214.
[57] J. Engelstädter, G. D. D. Hurst, *Ann. Rev. Ecol. Evol. Systemat*. **2009**, *40*, 127.
[58] J. Jaenike, K. A. Dyer, C. Cornish, M. S. Minhas, *PLoS Biol*. **2006**, *4*, e325.
[59] K. Koukou, H. Pavlikaki, G. Kilias, J. H. Werren, K. Bourtzis, S. N. Alahiotis, *Evolution* **2006**, *60*, 87.

[60] J. Buellesbach, C. Greim, R. Raychoudhury, T. Schmitt, T. Ethology, **2014**, *120*, 834.
[61] A. Telschow, M. Flor, Y. Kobayashi, P. Hammerstein, J. H. Werren, *PLoS One* **2007**, *2*, e701.
[62] T. Ikeya, S. Broughton, N. Alic, R. Grandison, L. Partridge, *Proc. R. Soc. B* **2009**, *276*, 3799.
[63] F. E. Champion De Crespigny, T. D. Pitt, N. Wedell, *J. Evol. Biol*. **2006**, *19*, 1964.
[64] A. Telschow, P. Hammerstein, *J. H. Werren. Evolution* **2005**, *59*, 1607.
[65] H. Laven, *Nature* **1967**, *216*, 383.
[66] A. Telschow, N. Yamamura, J. H. Werren, *J. Theoret. Biol*. **2005**, *235*, 265.
[67] C. L. Brelsfoard, Y. Séchan, S. L. Dobson, *PLoS Negl. Trop. Dis*. **2008**, *2*, e129.
[68] T. Tsuchida, R. Koga, T. Fukatsu, *Science* **2004**, *303*, 1989.
[69] J.-C. Simon, S. Boutin, T. Tsuchida, R. Koga, J.-F. Le Gallic, A. Frantz, Y. Outreman, T. Fukatsu, *PLoS One* **2011**, *6*, e21831.
[70] D. J. Hawthorne, S. Via, *Nature* **2001**, *412*, 904.
[71] T. E. Leonardo, G. T. Muiru, *Proc. Roy. Soc. B* **2003**, *270*, S209.
[72] T. E. Leonardo, E. B. Mondor, *Proc. Roy. Soc. B* **2006**, *273*, 1079.
[73] P. T. Leftwich, N. V. E. Clarke, M. I. Hutchings, T. Chapman, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E4549.
[74] J. A. Chandler, J. Morgan Lang, S. Bhatnagar, J. A. Eisen, A. Kopp, *PLoS Genetics* **2011**, *7*, e1002272.
[75] A. C. Wong, J. M. Chaston, A. E. Douglas, *ISME J*. **2013**, *7*, 1922.
[76] P. M. Miller, J. B. Saltz, V. A. Cochrane, C. M. Marcinkowski, R. Mobin, T. L. Turner, *PLoS One* **2011**, *6*, e16436.
[77] K. Jensen, C. McClure, N. K. Priest, *J. Hunt. Aging Cell* **2015**, *14*, 605.
[78] M. Archetti, F. Úbeda, D. Fudenberg, J. Green, N. E. Pierce, D. W. Yu, *Amer. Nat*. **2010**, *177*, 75.
[79] N. Lhocine, P. S. Ribeiro, N. Buchon, A. Wepf, R. Wilson, T. Tenev, B. Lemaitre, M. Gstaiger, P Meier, F. Leulier, *Cell Host Microbe*. **2008**, *4*, 147.
[80] J. H. Ryu, S. H. Kim, H. Y. Lee, J. Y. Bai, Y. D. Nam, J. W. Bae, D. G. Lee, S. C. Shin, E. M. Ha, W. J. Lee, *Science* **2008**, *319*, 777.
[81] A. Bost, S. Franzenburg, K. L. Adair, V. G. Martinson, G. Loeb, A. E. Douglas, *Mol. Ecol*. **2018**, *27*, 1848.
[82] C. Capuzzo, G. Firrao, L. Mazzon, A. Squartini, V. Girolami, *Int. J. Systemat. Evol. Microbiol*. **2005**, *55*, 1641.
[83] J. L. Feder, S. H. Berlocher, J. B. Roethele, H. Dambroski, J. J. Smith, W. L. Perry, V. Gavrilovic, K. E. Filchak, J. Rull, M. Aluja, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 10314.
[84] I. S. Pais, R. S. Valente, M. Sporniak, L. Teixeira, *PLoS Biol*. **2018**, *16*, e2005710.
[85] E. R. Hester, K. L. Barott, J. Nulton, M. J. Vermeij, F. L. Rohwer, *ISME J*. **2016**, *10*, 1157.
[86] J. A. Chandler, M. Turelli, *Science* **2014**, *345*, 1011.
[87] T. Chapman, *Am. Nat*. **2018**, *192*, 217.
[88] J. D. Shropshire, S. R. Bordenstein, *MBio* **2016**, *7*, e01785.
[89] L. Keller, H. K. Reeve, *Levels of Selection in Evolution*, (Ed: Keller L), Princeton University Press, **1999**, pp. 153.
[90] J. Jaenike, *Oecologia* **1983**, *58*, 320.
[91] D. Lachaise, M. L. Cariou, J. R. David, F. Lemeunier, L. Tsacas, M. Ashburner, *Evol. Biol*. **1988**, *22*, 159.
[92] L. J. Funkhouser, S. R. Bordenstein, *PLoS Biol*. **2013**, *11*, e1001631.
[93] A. Lizé, R. McKay, Z. Lewis, *ISME J*. **2014**, *8*, 469.