With a little help from my friends: the roles of microbial symbionts in insect populations and communities.

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Abstract:

To understand insect abundance, distribution, and dynamics, we need to understand the relevant drivers of their populations and communities. While microbial symbionts are known to affect many aspects of insect biology, research on their ecological and evolutionary importance for wild non-model insects is scarce. We are still far from understanding the spatio-temporal dynamics of symbioses in natural insect communities, symbiont effects on populations or communities, or on insects' evolutionary responses at different timescales. Also, we can only wonder how these effects change as the anthropogenic effects on ecosystems intensify. However, recent developments in sequencing and bioinformatics permit cost-effective microbial diversity surveys, tracking symbiont transmission, and identification of functions across populations and multi-species communities, helping us address these questions.

In this review, we explore how different functional categories of symbionts can influence insect biology at different levels, how these effects could alter interactions among species, and plausibly affect processes at the level of entire communities. We argue that insect-associated microbes should be considered as likely essential drivers of insect response and adaptation to environmental challenges and opportunities. We also outline the emerging approaches for surveying and characterizing insect-associated microbiota at population and community scales.

Keywords: microbiome, symbiosis, facultative endosymbiont, adaptation, *Wolbachia*, barcoding

Introduction

Insects are the Earth's most diverse group of eukaryotic organisms [1]. They live on all continents, inhabit most environments, and fulfill a diverse array of crucial roles for ecosystems and humans. However, the recent realization of the magnitude and pace of ongoing global insect declines has made it clear that we cannot take these services for granted [2]. The comprehensive and accurate characterization of the shifting patterns of insect diversity, distribution, and functions has indeed become one of the priorities of biological research in the era of anthropogenic changes.

However, to fully understand insect abundance, distribution, and dynamics, we must understand the relevant drivers of insect populations and communities. Among those least understood are insects' relationships with their symbiotic microorganisms. The tremendous diversity of insects is reflected by at least as diverse array of microbial symbionts. They have played pivotal roles in insect evolutionary success and diversification, especially in exploring new food niches [3,4]. Through their diverse effects on host life history traits, symbionts have played important roles in many aspects of insect biology, including responses to environmental challenges [5,6]. Through their effects on the biology of keystone species and the ability to transmit and express their effects across species, they are likely to affect entire communities [7,8]. In the face of ongoing, rapid environmental changes and global loss of insect biodiversity [2], understanding the role of microorganisms in insects' biology and adaptation is more important than ever.

Since the initial discovery of non-pathogenic microorganisms within insect tissues [9] until the turn of the last century, researchers have primarily relied on microscopy, later supplemented by experiments, to obtain insights into insect-microbe relationships [10]. However, DNA-based techniques' broad implementation and rapid development have dramatically sped up the discovery pace, providing new insights into microbial diversity, distributions, co-phylogenetic relationships with hosts, and genomic evolutionary patterns. DNA-based techniques have also provided the foundation for multi-pronged investigation of symbiont functions in several symbiotic systems.

Researchers have been approaching insect-microbe interactions from various angles (Fig. 1). More often than not, the primary focus of the investigation has been on the microbes: microbial community composition or microbial clades' distribution across host species or populations. Attention has also been paid to microbial effects on host life history traits and the molecular mechanisms of symbiosis. However, we generally lack an understanding of processes above the individual level. Despite their apparent potential, microbes are rarely considered a force capable of influencing insect populations and communities. We are far from answering broad questions about the symbionts' importance at ecosystem scales:

- How are natural populations of insects - their abundance, distributions, genetic diversity, patterns of interactions with other species - affected by microbial symbionts?
- How microbial symbionts affect species populations other than their hosts - what are the mechanisms, directions, and magnitude of effects, and how common are these?
- To what extent do microbiota affect wild multi-species communities' composition and function?
- Can microbes routinely serve as a means of transmission of ecologically relevant traits across insect species?
- Can species respond and adapt to environmental challenges and opportunities, including those of the Anthropocene era, through microbial symbioses?
- Can microbes significantly influence the processes and patterns of insect biodiversity declines?
- How can we use information about microbial symbioses to aid agricultural, biomedical, conservation, and other efforts?
- How can we plausibly and cost-effectively characterize and monitor symbiont communities and their effects at population and community scales?

This review aims to summarize our knowledge of the many ways and levels at which microbial symbionts could influence insects, explain how these effects can manifest within populations and communities, and outline plausible approaches for studying microbiota at such scales.

Figure 1. Insect-microbe symbioses can be studied from different angles. **A. Microbiome perspective** concerns microbial communities' composition and function across individuals, populations, or species. **B. Symbiont perspective** concerns specific microorganisms of particular importance in the biology of certain insects and focuses on their distribution, transmission, genomic evolution, or functions. A related but distinct level of investigation concerns mechanisms of host-symbiont interaction. **C. Host perspective** addresses questions about how insect life history traits and functions can be affected by symbiotic partners. **D. Population perspective** concerns symbiont effects on insect populations - performance, functions, genetic diversity, and evolutionary potential. **E. Community perspective** addresses questions about symbiotic host's interactions with other species in the community, and thus symbionts' effects on community processes, composition, and functions. This review aims to link symbionts' effects on insect hosts with population and community processes: we focus on levels C, D, and E.

Box A. Glossary

- **Symbiosis** - a close long-term relationship between different species, often from different kingdoms, regardless of the nature of interaction among the partners: mutualistic, commensalistic, or parasitic [11]. In insect-microbe symbioses, the insect partner is referred to as the **host**, and microbes - as **symbionts**.
- The terms **microbiota** and **microbiome** have often been used interchangeably. However, according to recent definitions, the term "microbiota" describes the assembly of microorganisms - Bacteria, Archaea, Protozoa, fungi, algae - inhabiting a well-defined habitat. At the same time, the term "microbiome" also encompasses their theatre of activity: microbial structures, metabolites, mobile genetic elements (e.g., transposons, phages, and viruses), and relic DNA [12].
- **Horizontal and vertical transmission** are two primary means of symbiont transmission across individuals. Vertical, or maternal symbiont transmission is from the mother to her offspring. Horizontal transmission occurs between unrelated individuals from the same or different species.
- **Endosymbionts** are symbiotic microorganisms living within the host body cavity or hemocoel (within hemolymph, tissues, or inside cells). Those living outside the body cavity (within the gut lumen, on the cuticle, or in various glands) are sometimes referred to as **ectosymbionts**. However, this last term can be confusing when referring to internally localized gut microbes, and hence, we recommend stating the body localization when talking about these microbes.

The functional diversity of insect symbioses

To understand the effects of microbes on insect biology, it is essential to be aware of the diversity of host-symbiont interactions, including the functional diversity of symbionts and the variation among insects in their reliance on symbionts. The traditional classification of insect symbionts was based on microbial localization in host tissues and on their effects on host biology. The first category are endosymbionts - residing within the body cavity - in hemolymph or intracellularly, within dedicated tissues [13], and forming either obligate nutritional or facultative associations. They are contrasted with microbes residing outside of the host body cavity - within the gut lumen or on the external cuticle. Recently, Perreau and Moran [14] proposed to classify symbioses as "Open", "Closed", or "Mixed", based on transmission mechanisms and the stability of host-symbiont associations. Hammer et al. [15] emphasized both the stability (specialized vs. transient) and the nature of the relationship (beneficial vs. harmful).

To consider symbionts' roles in insect populations and communities, it may be helpful to combine these classifications. Hence, <u>here we will refer to five broad symbiont categories that differ in their effects on insect biology and evolution.</u>

- **"Closed" nutritional symbioses** comprise obligatory endosymbionts such as in the sap-feeding hemipteran clade Auchenorrhyncha [16], and strictly maternally transmitted gut symbionts in insects such as *Cassida* leaf beetles [17]. Having enabled their hosts' evolutionary adaptations on nutrient-limited foods, these strictly heritable microbes are now essential to hosts. Generally regarded as stable over a long time, these associations

may change through symbiont replacement or complementation. Some of these recent changes have shifted host biology substantially [18,19].

- **"Mixed" facultative endosymbionts** comprise multiple clades of bacteria that inhabit insect haemolymph and tissues and can influence many of their life history traits, particularly those related to defense and reproduction [20–24]. Through such often major effects, combined with the ability to transmit both vertically and horizontally, they can spread rapidly within populations and move within communities

- **Host-adapted ectosymbionts** inhabit the gut lumen, cuticle, or insect's proximate environment, often forming multi-species communities [25,26]. They may be transmitted from parents to offspring or socially or acquired from the environment each generation. Hence, their co-evolutionary history with insects and distribution within and across species can vary.

- Microbial **pathogens** primarily transmit horizontally and, upon infection, usually negatively affect host function. Insects also serve as intermediate hosts and vectors of pathogens more damaging to other organisms, including plants and vertebrates [27,28].

- **Transient microbes** do not form stable associations with particular insect species but may be abundant in individuals, perhaps as a result of ingestion of microbe-rich food, surface contamination, or atypical colonization. As such, these microbes may often contribute to microbial community profiles, challenging the identification of microbes with more consistent infection patterns or effects on hosts.

Delimiting these categories, or assigning a particular microbe to one, can be challenging, with intermediate states in existence, ongoing evolutionary transitions among categories, and even closely related microbes falling into different categories [29]. For example, insect nutritional endosymbionts often seem to be derived from bacterial or fungal opportunists or pathogens in the genera *Sodalis*, *Ophiocordyceps*, and others [30–32].

Conversely, insect clades and species differ in how reliant they are, and how affected they are by microbial symbionts (Fig. 2). Strict dependence on the hosts is most often linked to nutrition. Insects dependent on specialized, co-adapted microbes for nutrition include clades specialized on nutrient-imbalanced or recalcitrant foods such as plant sap or blood. Association with bacteria that evolved into obligate endosymbionts providing deficient essential amino acids and vitamins allowed several clades to switch to and specialize on nutrient-limited diets, tens or hundreds of millions of years ago in some cases [33,34](Fig. 2A). These symbionts have co-diversified with hosts, occasionally undergoing replacement or complementation by bacteria or fungi soon converging on the same set of nutritional functions [18,35,36]. Insects may also rely on specialized endo- or gut symbionts for a different set of nutritional functions, including the provisioning of tyrosine in weevils [37], or pectinases in leaf beetles [17]. Likewise, we expect strict reliance in insect clades that harbor conserved multi-species gut microbiota that co-diversify with hosts. Pollen-feeding corbiculate bees, wood-feeding termites, herbivorous *Cephalotes* ants, and seed-feeding heteropterans all associate with gut microbiota socially or maternally transmitted for tens of millions of years and shown to address their hosts' specific nutritional needs [38–41](Fig. 2B). Many herbivores seem to rely on certain microbes for the degradation of specific toxins present in the diet [19,42]. A distinct type of mutually obligate nutritional association is between insects such as leafcutter ants, fungus-growing termites, and diverse wood-boring

beetles, which all associate with specialized fungi that they farm as the sole or primary source of nutrition [43–46](Fig. 2C).

A related category are insects dependent for nutrition on microbes but more versatile in their partner choice. Heteropterans [superfamily: Pentatomoidea] depend on nutrient provisioning for maternally transmitted gut bacteria, but different populations associate with distantly related bacteria [47], and even *Escherichia coli* can become a suitable symbiont following specific mutations [48]. Distinct patterns have been reported from coreoid and lygaeoid bugs, which rely for nutrient provisioning on *Caballeronia (*previously *Burkholderia*) symbionts, which each generation acquires from soil [49,50](Fig. 2D). The reproductive success of burying beetles, whose larvae develop in vertebrate carcasses, also rely on bacterial communities with somewhat variable composition [51,52] (Fig 2E).

Some insects depend on specific microbes for defense or reproduction. As discussed later, many insects benefit from microbe-conferred protection, but in some systems, host-symbiont co-diversification and anatomical and behavioral adaptation for symbiont transmission suggest strict dependence. An excellent example are beewolves, protected from soil-inhabiting fungal pathogens during the pupal stage by specialized, maternally transmitted Actinobacteria within cocoons [53,54](Fig. 2F). Leafcutter ants also rely on Actinobacteria (genus *Pseudonocardia*) for controlling fungal parasites threatening their symbiotic fungi [55]. In the psyllid genus *Diaphorina*, its symbiont *Ca.* Proftella bears many characteristics of an obligate endosymbiont, including the ability to produce vitamins, but a large portion of its reduced genome comprises genes for synthesizing diaphorin, a polyketide toxin providing wide-spectrum protection against natural enemies [56]. There are also known cases of insects' dependence on a symbiont for reproduction, as shown in the hymenopteran genus *Asobara* [57].

However, many insects do not depend on microbes, and infections are maintained through a balance of benefits and costs. Benefits are often linked to nutrition or protection against various natural enemies and abiotic stressors (Fig. 2G). Costs may be linked to the symbionts' maintenance or over-replication, but also reproductive manipulation (Fig. 2H). In the next section, we outline and discuss the diversity and significance of these non-essential effects.

Finally, some insects may only occasionally associate with microbes - or even not routinely host microbes. For example, ant genera differ by orders of magnitude in gut bacterial densities, and in many of them, bacterial levels are below the detection thresholds of standard techniques [58](Fig. 2I). Likewise, lepidopteran larvae (caterpillars) were shown to host, on average, over 10,000-fold fewer bacteria per gram of body mass than other animals used in comparisons [59]. In these cases, low gut bacterial abundances may be due to a reduced need for nutritional supplementation thanks to balanced diets, limited opportunities for microbial colonization, and/or effective microbial control mechanisms. Many other insects may be in a similar position [15], but estimates of numbers are lacking. Also, insects with virtually no specialized gut bacteria may still be prone to at least occasional colonization by facultative endosymbionts, pathogens, or transient microbes.
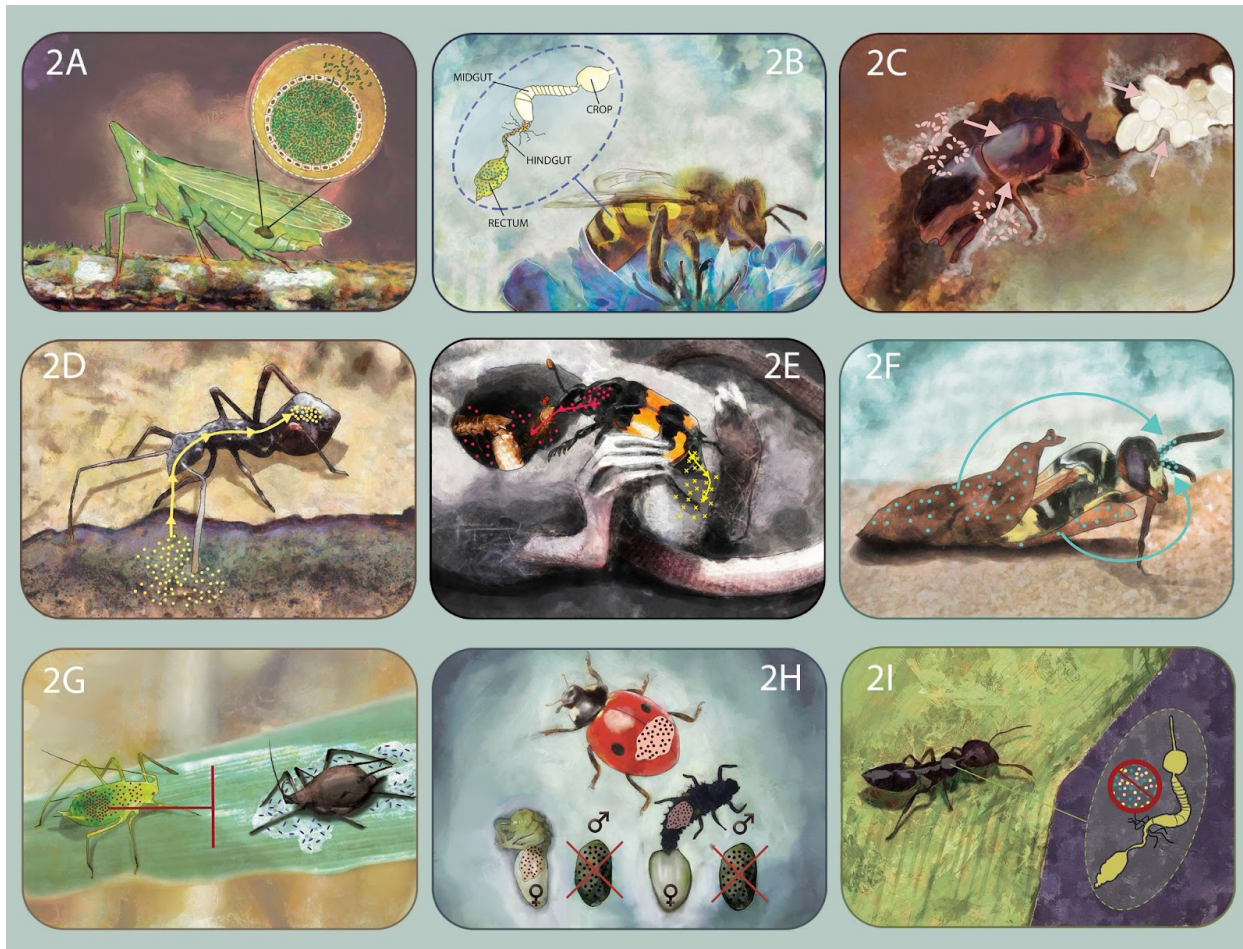
Figure 2. Examples of insects that rely on symbionts to different extent and for different functions. A. Planthoppers are among sap-feeding hemipterans that cannot survive without nutritional supplementation from specialized microbes that live within dedicated insect tissues and are transmitted transovarially across generations. **B.** Many insects rely on complex, structured, reliably transmitted gut microbial communities that provide nutritional and defensive functions. In honeybees, such gut microbiota date back at least 100 million years. **C.** Ambrosia beetles are among the species that culture specialized fungi. Inoculated within tunnels that beetles construct in living trees, fungi are not only critical to overwhelming tree defenses but also serve as the beetles' only food source. **D.** Some insects depend on microbes that they acquire from the environment each generation. In *Riptortus* bean bugs, *Caballeronia* symbionts, acquired by nymphs from the soil, provide nutrients and can confer other benefits, including pesticide detoxification. **E.** Controlling microbial community in the environment can be essential for nutrition and safety. Burying beetle adults inoculate carcasses that their larvae develop in with their microbiota and provide them to larvae while spreading antimicrobial compounds that help control harmful bacteria. **F.** Insects' reliance on protective symbionts can lead to specific adaptations and long-term co-diversification of partners. In beewolves, *Streptomyces* that protect cocoons from fungal pathogens are transmitted within dedicated antennal glands. **G.** Symbionts often protect insects against a variety of environmental challenges. For example, several facultative endosymbionts protect pea aphids against a specialized fungal entomopathogen. **H.** Insects commonly have their reproduction affected by symbionts - benefitting microbes, but not

necessarily the hosts. For example, in a ladybird *Adalia bipunctata*, transovarially transmitting facultative endosymbionts kill male embryos, resulting in more resources for females. **I.** Many insects do not seem to rely on specialized microbes. Ants in the genus *Crematogaster* are among those lacking observable amounts of microbiota within their digestive tract.

How non-essential symbionts affect insect life history traits

Symbioses essential to hosts, described in the previous section, may not necessarily be fixed, and symbiont replacement could provide hosts with novel capabilities and functions [3,6]. For example, a swap of a specialized gut symbiont in the kudzu bug (*Megacopta cribraria*) with a related species led to the ability to feed on soybeans, resulting in agricultural pest status in its introduced range [60].
However, symbionts that form facultative, non-essential associations with insects can express their effects on insects' life history traits and biology in a much more dynamic manner. We can group these effects into three primary areas: nutrition, defense, and reproduction. Within each of these areas, one could distinguish multiple categories. We have listed some of these effects in Table 1, and discuss some of the mechanisms, patterns, and consequences below.

Microbial symbionts' effects on host nutrition can be facultative but potentially have significant effects. Aphids are able to feed on plant sap thanks to nutritional contributions of obligatory endosymbionts, but in some pea aphid populations, facultative endosymbionts were shown experimentally to improve fecundity on particular plant species [61,62]. Similar patterns were recently reported from cowpea aphids infected with *Arsenophonus* [63]. Then, symbionts can reinforce dietary specialization, potentially leading to reproductive isolation and speciation, but also enable expansion or shift of a dietary range [64]. In several systems, symbionts may contribute to nutrition in specific ways. For instance, in *Drosophila* fruit flies and honey bees *(Apis mellifera)*, larvae can escape the results of undernutrition through symbiosis with various bacterial symbionts [65,66]. Also, some symbionts may have limited effects under optimal nutrition but benefit hosts when conditions deteriorate. In *Drosophila melanogaster*, *Wolbachia*, involved in iron metabolism, increases the fecundity on diets with suboptimally low or high iron concentrations [67].

Among insect symbionts' most striking effects is the protection they confer against natural enemies. In multiple insect systems, different symbiont clades were shown experimentally to protect against predators, parasitoids, entomopathogenic nematodes, fungal entomopathogens, parasitic microeukaryotes, bacterial pathogens, and viruses [20–23] (Table 1). These effects can be manifested at different levels. First, symbionts can help insects evade the natural enemy attack by altering the production of chemical cues that parasitoids and predators use to locate their prey in complex environments and assess their suitability. For example, aphid symbionts may disrupt the production of parasitoid-attracting volatiles by plants that aphids feed on [68]. Symbionts may also affect the natural enemy choice, for example, by discouraging them from ovipositing [69]. In aphids, some facultative endosymbionts may delay host deaths caused by entomopathogenic fungus and make them fall off plants prior to sporulation, thus reducing the exposure of their clonal kin to infectious spores [70].

Further, symbionts can aid their hosts by decreasing the success of an attack or infection. Possible mechanisms include altering cuticle properties, especially thickness and density [71,72], the attacked insects' defensive responses [73], or its overall condition that may determine the effectiveness of the defense.

However, symbionts' effects on their insect abilities to fight off infections are best known. Insect-associated microbes have been shown to encode diverse biosynthetic pathways and produce a wide range of defensive compounds and toxins, often with demonstrated defensive properties [74–76]. Another protective mechanism could be stimulating the host's immune system or playing a role in its maturation [77,78]. Further, symbionts can also protect their hosts by competing for host resources that are vital for the pathogen [79].

Insects can also enjoy symbiont-conferred protection against a wide range of environmentally relevant abiotic stressors. Facultative endosymbionts have been shown to protect their hosts against heat [80–83], water loss and desiccation [72], pesticides [84], and heavy metals [85] (Table 1). In all cases, experiments showed a clear increase in survival or reproductive fitness-related traits of the exposed individuals that carried protective symbionts.

Another important category of symbionts' effects is reproductive manipulation [86]. Facultative endosymbionts representing divergent bacterial clades alter the reproduction of their insect hosts to maximize their maternal transmission and facilitate spread in a population. There are four distinct strategies of reproductive manipulation, each induced by strains of several different bacterial genera, and each reported from a wide range of hosts [24]. The feminization of genetic males leads to their development into functional females that can pass on the infection to their offspring. Male killing leads to the death of male embryos, increasing the share of the local resource pool available to females and translating to their improved survival, growth, and, ultimately, reproductive output. Another strategy is parthenogenesis induction, where unfertilized eggs develop into females. Finally, the most common strategy may be the induction of cytoplasmic incompatibility - where embryos sired by symbiont-infected males suffer high mortality unless the same symbiont strain also infects the mother. This reduces non-infected females' reproductive success as the symbionts' prevalence in the population increases.

All these effects, while benefitting the symbiont, generally happen at the expense of at least some components of the insect fitness: the reproduction through females is generally improved, but the reproduction through males suffers [86]. Symbionts can also have more nuanced reproductive effects, such as altering the induction of sexual reproduction in cyclically parthenogenetic aphids [87].

Symbiont also affects host life history traits directly related to reproductive fitness, including juvenile survival, time to reproduction, lifespan, and fecundity. These effects can range from highly detrimental to strongly positive [88], depending on environmental conditions, genotypes, and likely other effects. Finally, other symbionts' effects may be harder to categorize but could strongly influence individuals and populations. For example, at low population densities, aphids tend to develop into wingless morphotypes with shorter development time and higher fecundity but limited mobility, while under crowded conditions, they are more likely to develop into less fecund-winged

forms [89]. Facultative endosymbionts can decrease aphid sensitivity to crowding in regard to winged morphotype induction, thus decreasing their propensity to migrate [90].

There are multiple considerations when assessing symbiont effects on insect life history traits. Microbes often simultaneously incur different effects on their hosts. An excellent example is the combination of reproductive manipulation and antiviral effects conferred by different strains of *Wolbachia*, which led to its widespread adaptation for controlling mosquito-vectored dengue or related viruses [91]. Symbionts' phenotypic effects can often be expressed in different host genotypes and species. For example, facultative symbionts and their effects can be artificially transmitted across aphid and *Drosophila* clonal genotypes and species [92,93]. Likewise, the use of *Wolbachia* wMel for dengue control is possible thanks to its similar reproductive and antiviral effects in original (*Drosophila*) and new (*Aedes*) hosts [91]. On the other hand, the symbiont effects on hosts can be highly specific, with variation reported among genotypes and species of symbionts, their insect hosts, and natural enemies that symbionts protect from [94–96]. The diversity of fungus- or parasitoid-protective facultative endosymbionts in pea aphid populations was explained by the specificity of their protection against diverse natural enemy genotypes present in the environment [70]. Specificity was also demonstrated in reproductive manipulation, with host genotypes varying in susceptibility to manipulative symbionts [97], and cases where the same symbiont strain manipulates alternative hosts in different ways [98].

Symbiont effects on hosts can also depend on environmental conditions. For example, parasitoid protection conferred to aphids by *Hamiltonella* can falter at higher temperatures [99]. Similarly, it has been shown that with higher temperatures, *Wolbachia* confers lower protection against viruses [100]. Environmental conditions can also affect host-symbiont interactions indirectly [81]: elevated temperatures can reduce *Wolbachia* maternal transmission efficiency [101], leading to the loss of any protective or reproductive effects it may have. Last but not least, symbionts can alter each other's effects in co-infections. In aphids, *Fukatsuia* extends the thermal range of *Hamiltonella*-conferred parasitoid protection [82], *Hamiltonella* may ameliorate negative fecundity effects of *Rickettsia* without decreasing its fungal protection strength [92], and *Regiella* and *Hamiltonella* influence each other's defensive and fecundity effects in complex ways that vary among host genotypes [102]. Symbionts can also affect each other's transmission efficiency [103].

Effect	Examples
A shift in performance on alternative diets and extension of the dietary range	*Arsenophonus* in the cowpea aphid [63] *Enterococcus* in armyworm (*Spodoptera frugiperda*) caterpillars [104] The microbiome of the Colorado potato beetle (*Leptinotarsa decemlineata*) suppresses antiherbivore defenses in tomato plants [105]
Supplementation on suboptimal diets	*Bombella apis* in honey bee larvae [66] *Lactobacillus plantarum* in *Drosophila* larvae [65]
Protection against predators	*Pseudomonas* in rove beetle *Paederus sp.* [106] Decreasing of predators' fitness by facultative symbionts of pea aphids

	[107,108]
Protection against fungal pathogens	*Regiella* and other facultative endosymbionts in the pea aphid [70] *Burkholderia* in the cuticle of *Lagria* beetles [109] Facultative endosymbiont *Rickettsia* in whiteflies [110] *Streptomyces* in beewolf cocoons [54]
Protection against parasitoids	*Hamiltonella* in aphids [95] *Spiroplasma* in *Drosophila hydei* [111]
Protection against entomopathogenic nematodes	*Spiroplasma* in *Drosophila neotestacea* [112,113] Fungal symbiont in *Sirex* wood wasps [114] Scarabid beetle (*Melolontha melolontha*) microbiome targeting mutualistic symbionts of nematodes [115]
Protection against microeukaryotic parasites	Bumblebee gut microbiota against trypanosomatid *Crithidia bombi* [116]
Protection against bacterial pathogens	Larval microbiota in Japanese honeybees (*Apis cerana japonica*) against bacterium *Paenibacillus larvae* [117] *Morganella* and *Providencia* provide resistance against *Serratia*-induced mortality in carrion beetles [51]
Protection against viruses	*Wolbachia* in *Drosophila* [118], mosquitoes [119] and brown planthopper (*Nilaparvata lugens*) [120]
Protection against heat shock	*Regiella, Fukatsuia* [82] and *Serratia* [83] in pea aphid (*Acyrthosiphon pisum*) *Wolbachia* in *D. melanogaster* [121]
Protection against pesticides	Soil-acquired *Caballeronia* in the bean bug and other related bugs [84]
Heavy metal detoxification	*Snodgrassella alvi* and *Lactobacillus bombicola* in bumblebees [85] Egg microbiome in chironomids [122]
Overall increase in reproductive fitness	Facultative endosymbiont *Rickettsia* in whiteflies [123] *Wolbachia* wRi in *Drosophila simulans* [124,125]
Reproductive manipulation	Diverse facultative endosymbionts including *Wolbachia*, *Rickettsia*, *Spiroplasma*, and *Cardinium*, induce feminization, male killing, parthenogenesis, or cytoplasmic incompatibility in a wide range of insect clades [86,126]
Induction of dispersal	*Regiella* reduces winged morph induction under crowded conditions in pea aphids [90]

Table 1. Examples of significant effects of non-essential microbial symbionts on life history traits of their insect hosts

Box B. *Wolbachia* as a widespread symbiont clade, affecting insects in a variety of ways

The alphaproteobacterial genus *Wolbachia* is the most broadly distributed insect symbiont, with at least 17 recognized clades ("supergroups") colonizing diverse insects, other arthropods, and filarial nematodes [127]. *Wolbachia* infects about half of all insect species, but the infection prevalence varies among and within species - across populations and over time [98,128,129]. Its associations with insects range from facultative to obligate, covering the spectrum from parasitism to mutualism.

Wolbachia has historically been regarded as a reproductive parasite, manipulating hosts' reproduction in four distinct ways: cytoplasmic incompatibility, male killing, feminization of genetic males, or parthenogenesis induction [86]. Manipulation aids symbiont spread within host populations but negatively affects at least some aspects of the hosts' fitness [129]. In some cases, exemplified by a hymenopteran parasitoid *Asobara tabida*, its tight integration into host oogenesis has made it necessary for reproduction - a sort of indispensable parasite [130]. At the same time, *Wolbachia* can have important beneficial effects. In bed bugs, it is an essential nutritional mutualist providing B vitamins required by these obligatory blood feeders [131]. More subtle and non-essential nutritional benefits were reported from *Drosophila melanogaster*, through the symbiont's apparent effects on iron metabolism [67].

Wolbachia has also become known for its defensive effects, especially against viruses [118,132]. These anti-viral effects are the foundation of extensive experimental work on *Wolbachia*-driven control of mosquito-vectored dengue and related viruses [91], or planthopper-vectored plant viruses [120]. Recently, *Wolbachia* was also shown to protect aphids against a fungal pathogen [133]. Finally, it has been linked to host thermal preferences and performance, as suggested by geographic gradients in its prevalence [101].

Like other facultative endosymbionts, *Wolbachia* transmits mainly maternally (vertically) with high fidelity but is also capable of horizontal transmission - within and across species [98]. The presence of nearly identical strains in different host species [134] and the lack of congruence between host and symbiont phylogenies [135], highlight host switching as an important aspect of *Wolbachia* biology, leading to the transmission of its phenotypic effects across host species [98]. The combination of reproductive manipulation, fitness benefits, and alternative transmission means thus provides avenues for *Wolbachia*'s rapid spread within and among species [134,136], affecting population- and community-scale processes and patterns.

How symbionts affect insect populations and species

Symbiont effects on individual life history traits were generally demonstrated under controlled laboratory conditions that aimed to limit the effects of other variables. Despite the uncertainty of how consistent the effects are in the wild, the results indicate how symbionts may influence host populations and the evolutionary trajectories of species on longer timescales.

Generally, once a heritable symbiont with a significant positive net effect on fitness is established in a population, we would expect the infection to spread alongside its ecologically relevant effects on hosts [137]. These processes may lead to structuring beneficial symbiont infections alongside

environmental gradients relevant to symbiont effects, such as natural enemy pressure, temperature, or available food [81,101,110,138]. Further, in species with short generation times, seasonal changes in environmental conditions and pressures should alter the balance of infection costs and benefits for different symbionts, likely promoting different types of infections at different times of the year. This could lead to seasonal fluctuations in the prevalence of different symbionts within populations [139–142]. These processes could lead to local or seasonal spread of adaptive phenotypes, increasing the overall population performance under specific conditions. Because of their effects, symbionts - especially facultative endosymbionts - have been described as a pool of horizontally transmitted vectors of ecologically significant traits that enable rapid response and adaptation of species to environmental challenges and opportunities [7,8]. The symbiont's primary fitness benefits, plus any additional effects on other life history traits, could rapidly shift the host species' ecological niche [3].

In the case of symbionts that primarily transmit maternally, symbiont spread within a population occurs through improved performance of host lines that carry certain symbionts. This changes the population's genetic makeup, leading to a symbiont-driven decrease in the host genetic diversity in a population [113,143,144]. In some cases, this could lead to selective sweeps, where symbiont-carrying host lines representing a small proportion of the initial genetic diversity dominate the populations. Loss of genetic variation may negatively affect insects' ability to respond to the changing environment through recombination and natural selection acting upon their own genomes [145]. Barriers to within-population gene flow caused by reproductive manipulation may have similar detrimental effects on the hosts' adaptation potential through their nuclear genome evolution. Further, the reproductive manipulation-driven decrease in the host's effective population size would increase the impact of stochastic processes. In the longer term, such symbiont-induced barriers to gene flow could facilitate and drive speciation [146,147]. On the other hand, symbionts' can also influence insect evolution through horizontal gene flow, whether serving as the original source of bacterial genes integrated into the host genome or by mediating gene transmission among different organisms [148].

The link between symbionts' effects on individual life-history traits and their effects on host lines' competitive performance and population structure was shown experimentally using semi-controlled population cages. By monitoring experimental populations started using a combination of insect lines carrying different symbionts, researchers have demonstrated in systems such as *A. pisum*, *B. tabaci, D. melanogaster,* and *D. neotestacea* how symbionts' protective effects lead to the spread of the infection in the presence of natural enemies, but not in their absence [110,149–151].

Patterns consistent with directional selection for maintaining certain symbionts were also reported from natural insect populations sampled across environmental gradients. For example, aphids from more northerly regions of Japan, characterized by higher humidity and thus more favorable conditions for fungal pathogens, carried pathogen-protective facultative endosymbiont *Regiella* more often [138]. On the other hand, comprehensive symbiont-monitoring efforts in the Eastern U.S. have made it clear that these symbioses are more dynamic. The prevalence of facultative symbionts in pea aphid populations changed throughout the season, sometimes, but not always, correlating with environmental pressures the symbionts protect against [139,140,142](Fig 3A).

This suggested that the symbiont prevalence is determined by a complex balance of infection costs and benefits, fluctuating over time in response to natural enemy pressures, temperatures, and other variables.

Strong symbiont-induced directional selection was also reported from several other systems. Over the last two decades, facultative endosymbiont *Spiroplasma* that protects fungivorous *Drosophila neotestacea* against an important natural enemy, the entomopathogenic nematode *Howardula aoronymphium*, has spread across the northern U.S. (Fig. 3B)[112,113]. The symbiont spread was linked to substantial changes in the population structure - shifts and the overall decrease in the relative abundance of mitochondrial variants across the surveyed range. Similarly, anti-fungal properties seem to have driven a rapid spread of *Rickettsia* across Chinese populations of the sweet potato whitefly *Bemisia tabaci*, following the spread of the entomopathogenic fungus the symbiont protects against [110]. In turn, the spread of *Rickettsia* in invasive whiteflies from the southwestern U.S. [152], seems to have been driven by a combination of reproductive manipulation and positive effects on female fecundity and development time [123].
Non-heritable symbionts can also alter host biology and performance in response to human-induced pressures. *Caballeronia s*trains acquired from the soil by juvenile stinkbugs from pesticide-treated areas frequently have the ability to detoxify these pesticides, making their hosts resistant and thus leading to symbiont-induced resistance within populations [84]. The long-term evolutionary consequences of these processes for the insects - including their status as agricultural pests - are not currently clear. There is also less data on the patterns, mechanisms, and ecological and evolutionary consequences of symbiont-induced pesticide resistance reported from other insect systems [153].

In the longer term, among the symbionts' most notable effects on insect evolution is driving their speciation. Among the best-researched examples is the Central- and South-American *Drosophila paulistorum* species complex, which comprises multiple reproductively isolated but often sympatric "semi-species" [146]. It was shown that the isolation among them is caused by *Wolbachia*, with different strains driving pre- and post-mating isolation between semi-species. This seems to be done through the symbionts' effects on host gene expression, altering pheromone production and reception [147].

Notably, symbionts' effects on host populations and species evolutionary patterns have been reported either from the best-studied dipteran genus or from hemipteran agricultural pests. We can be confident that similar effects play a role in species' biology that are more difficult to work with experimentally and less directly relevant to humans. Given the broad distribution of *Wolbachia* and other symbioses across insects, it seems plausible that processes such as symbiont-mediated spread of resistance to novel pressures or expansion of ecological niches or ranges are common aspects of insect evolution.
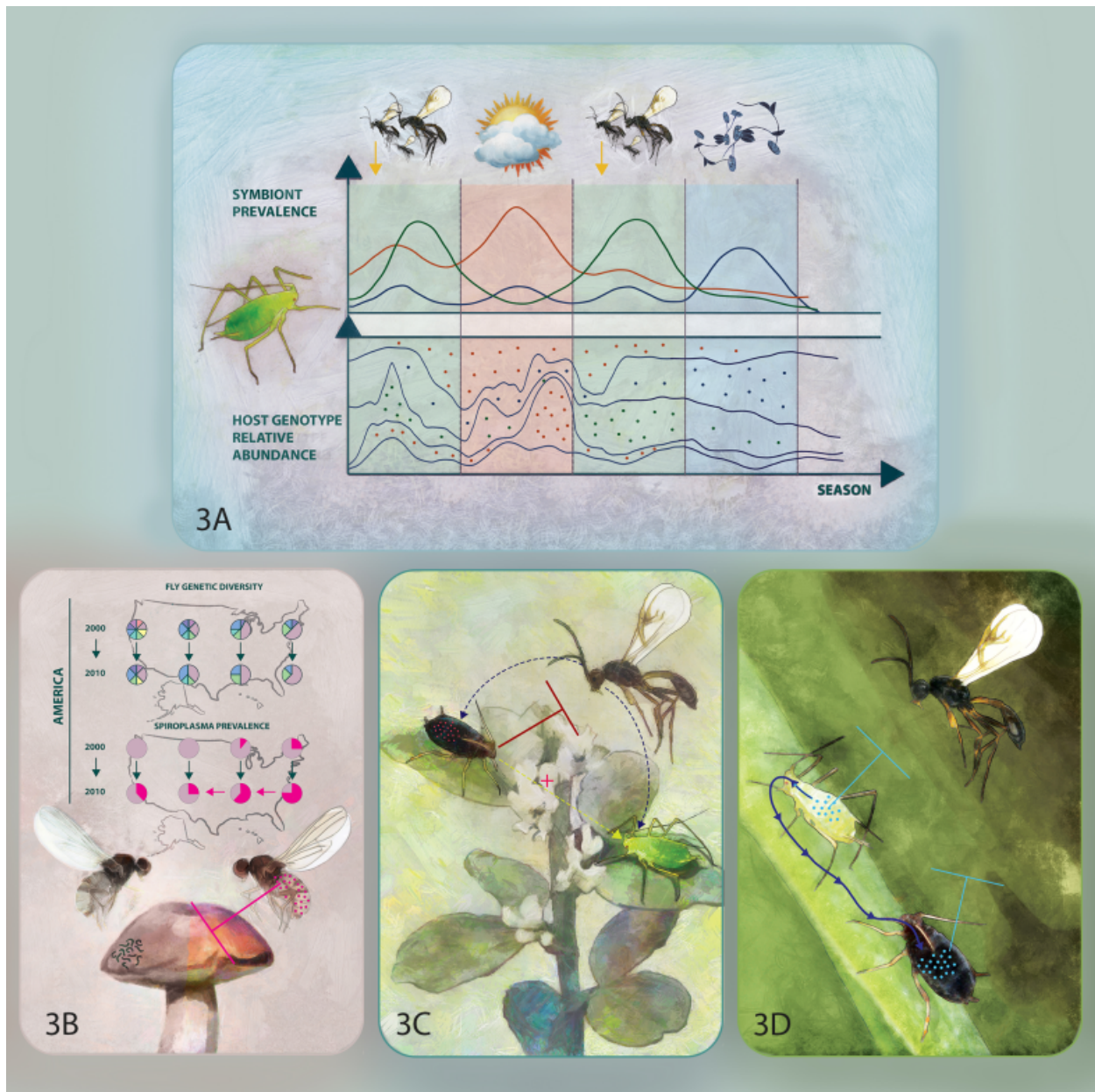
Figure 3. Examples of symbiont effects on insect populations and communities. A. Environmental pressures that aphid facultative endosymbionts can protect against, including the pressure of parasitoids, pathogens, and heat, vary throughout a season, promoting certain symbiont associations. The shifting prevalence of alternative symbionts affects the competitive balance among clonal lineages that carry them, affecting population structure. **B.** Rapid, continent-wide spread of nematode-defensive *Spiroplasma* symbiont in mushroom-feeding *Drosophila neotestacea* has resulted in the loss of host genetic diversity across populations. **C.** Defensive symbiont can protect their hosts directly but also have a range of indirect effects on the same or other species. For example, they can negatively affect populations of parasitoids that also attack other species, thus indirectly protecting these species. **D.** Facultative endosymbionts can transmit horizontally among species in a community and express the same effects in novel

hosts. Hence, transmitting a parasitoid-protective symbiont to a new host species can make that species also resistant to parasitoids.

Symbiont effects within multi-species communities.

We expect that the symbionts' effects on insect species' biological characteristics, population structure, abundance, or distribution may also influence predators, parasites, parasitoids, competitors, and other organisms with which the focal species interact. These effects can be cascading, influencing further species across the trophic network, with the infection effects potentially resonating within multi-species communities [7,8].

Among the most intuitive indirect symbiont effects are those that protective symbionts have on their hosts' natural enemies. Symbiont-conferred protection should decrease natural enemies' performance, negatively affecting their population processes. Indeed, in simple experimental communities, introducing defensive symbionts has led to the decline and, ultimately extinction of entomopathogenic nematodes attacking *D. neotestacea* flies [150]. We could expect the same types of effects against other categories of natural enemies.

However, the outcomes can be complicated by the specificity of protection. In aphids, strains of their defensive endosymbiont *Hamiltonella* vary substantially in the degree of protection they confer against parasitoid genotypes and species [154,155]. A high prevalence of a symbiont conferring an effective protection against a particular parasitoid species should lead to the decline of that parasitoid unless compensated by its rapid behavioral or genetic responses. Conversely, the shifting abundance or pressure by different parasitoids may change the adaptive value of hosting alternative defensive symbionts and, therefore, their prevalence in the host population. We would often expect negative density-dependent selection to occur: an increase in pressure by a certain parasitoid genotype would promote the spread of symbiont genotypes that protect against that particular natural enemy, which would then lead to its decline [137,139].

Analogously, symbionts could affect other types of interactions among host insects and other organisms. If a symbiont influences its herbivore host's feeding on a certain food plant - whether directly or indirectly, by altering the plant's anti-herbivore defenses [156] - changes in infection patterns within the herbivore population could plausibly affect the plants' performance and in the longer term, abundance. This would alter the adaptive value of hosting the symbiont for the herbivore, influencing infection prevalence, the herbivore's dietary range, and the pressure on the food plant.

However, species in a community are typically attacked by multiple natural enemy species - and correspondingly, predator or parasitoid host ranges frequently encompass multiple insect species [157]. Then, a defensive symbiont in insect species A that suppresses its natural enemy may indirectly protect species other than A that are attacked by the same natural enemy [150] (Fig 3C). On the other hand, we could also envision a behavioral shift in preference of the natural enemy towards species other than A, and an increased pressure on them. Either way, substantial

symbiont-mediated indirect effects on populations of other species will affect an even broader range of species that interact with them. That way, the effects of a defensive symbiont in just one ecologically significant species could well reverberate in the whole community. These processes have been demonstrated in simple experimental communities comprising different aphid and parasitoid species. Introducing a defensive symbiont into one of the aphid species has substantially affected populations of other species, sometimes leading to their extinction and the subsequent cascading community collapse [158]. We would expect such effects to be more subtle in spatially complex and heterogeneous natural ecosystems. Nevertheless, if the biology of keystone or outbreaking species is significantly affected by symbioses, one would expect substantial effects at the community level.

Food plants of herbivorous insects can also mediate symbionts' indirect effects on communities. The alteration of plants' anti-herbivore defenses by herbivores' symbionts could affect the performance of both the original herbivore and other herbivorous species [159]. Changes in the symbioses of one herbivorous species may thus well affect other species feeding on the same plant. Further, by producing volatile compounds, plants can communicate with each other, leading to the induction of anti-herbivore defenses in plants other than the one originally attacked. Likewise, through volatile production, plants can attract herbivores' natural enemies [68]. By attenuating the production of these volatiles, herbivores' symbionts can thus indirectly protect not only their own hosts but also alter the entire herbivore and natural enemy community in the vicinity [68].

A completely different take on symbiont roles in natural ecosystems is by considering their horizontal transmission within multi-species communities. As discussed, extensive phylogenetic evidence exists for facultative endosymbionts' transmission both within and across insect species, and they can express their diverse effects in a range of host genetic backgrounds. This has led several authors to put forward the view that facultative endosymbionts should themselves be regarded as the community, at least partly independent from the communities of insects that they colonize [7,8] (Fig 3D). Such symbiont communities could be regarded as pools of horizontally transmitted agents of ecologically relevant functions that insect species could subsample in response to various environmental pressures. They have been compared to plasmids in bacterial communities - exchanged among lineages and species, inherited by daughter cells, and encoding a variety of ecologically important functions [160].

This intriguing perspective aligns well with observations for systems such as aphids and *Drosophila* flies. Their facultative endosymbionts can be transmitted artificially, even among distantly related species [93,161], and there are indications of symbiont transmission within and among species on ecological timescales [162]. Symbiont prevalence fluctuates within populations and species, apparently due to the seasonally shifting balance of costs and benefits of infection with different strains [140]. On the other hand, we lack evidence on how freely aphid facultative endosymbionts move within natural communities. In particular, we do not have high-resolution phylogenetic data that would directly confirm very recent or ongoing symbiont transmission across species in the wild or of a newly acquired symbiont spreading naturally in aphid populations.

Another system that supports the perspective of a horizontally transmitted symbiont pool is *Wolbachia* in *Drosophila* flies. The discovery of nearly identical strains in species separated by tens of millions of years of evolution and from different corners of the globe strongly suggests that these symbionts do indeed transmit within communities at relatively short timescales [134].

For other insect-symbiotic systems, we have fewer indications that interspecific facultative endosymbiont transmission may occur at ecologically relevant timescales, especially across insect species that are more distantly related and functionally less similar. Recent genome-level phylogenies for over 100 *Wolbachia* strains and their diverse insect hosts do indeed reveal extensive symbiont transmission as species evolved [135]. The patterns observed in this study could have resulted from symbionts typically co-diversifying with hosts for millions or even tens of millions of years before potentially "jumping ship". On the other hand, one could also imagine the same patterns resulting from symbionts transmitting among species on a yearly basis. We cannot reliably distinguish among these scenarios without comprehensive and systematic additional sampling, both within insect populations and species and across communities.

We think that insect-associated bacteria within natural ecosystems, even those in a single functional category, do in fact, represent a large gradient of host specificity and abilities to transmit among species. It is plausible that instead of a single symbiont pool available to most members of the insect community, multiple sub-pools transmit more freely among related, or ecologically similar, or closely interacting species. However, in at least some systems, the inter-species transmission of symbionts and ecologically relevant traits that they encode does seem to occur on ecologically relevant timescales.

Symbionts as an unexplored means of rapid adaptation to environmental and anthropogenic challenges

The wide range of microbial symbionts' fitness effects, combined with the ability of many of them to transmit maternally and horizontally, justify their identification as likely means of rapid response and adaptation to a range of environmental pressures. This includes natural pressures associated with environmental gradients, such as those of temperature or rainfall, or seasonally variable drivers, such as the impacts of natural enemies. However, this also includes pressures that have appeared or intensified due to human activities. Global climate change alters local weather patterns and increases the incidence and severity of extreme weather events such as heat waves and drought episodes [163]. The production and environmental release of toxic agricultural chemicals continues going up on a global scale despite local declines [164]. The continuing destruction, degradation, and fragmentation of natural habitats is taking away living space for many organisms and, at the very least, disrupting species interaction networks [165,166]. Invasive species also adversely affect habitats and pressure native species through various means [167,168]. All these pressures have been listed among the most important drivers of the ongoing insect biodiversity declines [2,169]. At the same time, as discussed in the previous sections, insect symbionts can confer protection against most of these pressures in at least some insect systems.

Species can respond to novel or intensifying pressures in different ways. Responses can include behavioral changes or range shifts. Also, we would expect genome evolutionary processes, including random mutations and recombination, combined with genetic drift and natural selection, to play an important role [170]. However, these powerful processes are slow relative to the pace of the ongoing global changes. Considering this, it is interesting to consider the potential for rapid adaptation by acquiring symbiotic microorganisms, especially facultative endosymbionts. Such infection can lead to the near-instantaneous acquisition of complex, multi-gene traits, often of direct environmental relevance [3,6,7]. The effects of the new infection may be largely independent on gene sets or traits already possessed. The change may be heritable. Following such infection, the entire host-microbe symbiosis becomes the subject of selection - with the symbiont spreading in the population and the community alongside its host line. Such symbiont-driven adaptation could be more rapid and in-depth than the "textbook" evolutionary processes. In species subjected to very strong environmental pressures, one could imagine the spread of a protective infection to a high frequency within a few generations. Following the spread, evolutionary processes acting upon the symbiont genome - much faster than those affecting the host genome, could fine-tune and improve the symbiosis further [5,124]. Also, the increased local prevalence of a protective symbiont would likely increase the odds of its horizontal transmission within species or the colonization of new host species within the community.

A confirmation that such symbiont-driven rapid adaptation indeed frequently occurs in natural ecosystems would substantially change our understanding of community function and undergoing processes, especially in the face of the intensifying and speeding-up global changes. It could also open up new avenues for species management and conservation in agricultural or natural systems. But are such processes indeed common? Is it likely that the rapid continent-scale symbiont sweeps reported from *Drosophila* species [113,125] and whiteflies [110,152] are, or are becoming, a common occurrence affecting a wide range of species?

There are multiple questions and unknowns about such processes. We do not understand the breadth, structure, or nature of the symbiont pool that could plausibly be "sampled' by wild species, have a reasonable chance of colonizing, and plausibly form stable infections [8]. We are aware of the primary barriers to symbiont acquisition [171] but do not know their actual importance in natural systems. Through all these, we have no knowledge of how often symbionts such as *Wolbachia* may successfully colonize populations of new hosts and get a chance to spread: weeks or millennia? We do not know what are the most common features and effects of successful spreaders - perhaps a combination of defensive properties with reproductive manipulation?
Further, for symbionts that establish a foothold in a population, we do not know what are the typical fates of these infections. How common are rapid, selective sweeps, as opposed to slow increase to intermediate prevalence or co-existence of multiple strains at low prevalence in the population? How important are the interactions with any older symbionts? What happens after a successful sweep - is it common that the fitness benefits gradually dissipate [172]?

We cannot satisfactorily address these questions currently. Generally, our understanding of the ecological and evolutionary significance of symbioses in natural ecosystems is greatly hampered

by very limited data on the biodiversity of insect-microbe interactions. To get an accurate picture of the relevant processes and patterns, we do need to embark on a much more systematic characterization of insect-microbe interactions in the wild than has been achieved so far.

How to incorporate microbiome characterization in insect biodiversity surveys?

Studying microbiota in large numbers of wild insects requires a well-thought-of strategy for laboratory work, besides the specimen sampling strategy itself. Specifically, we want:

- Means of obtaining information about the taxonomic identity of the processed insects and, preferably, the presence and identity of parasitoids or parasites that could confound the microbial community profiles.
- Data on the presence, absolute abundance, and taxonomic identity of microbes that may colonize the insects - bacteria, but ideally also fungi, microeukaryotes, and viruses.
- The ability to obtain high phylogenetic resolution for selected hosts and microbes and information about their functions.
- Labor- and cost-effectiveness of the laboratory workflow so that it is plausibly applicable to hundreds or thousands of individuals at a time
- The microbiome-focused workflow should be integrated with the insect biodiversity surveying workflow, enabling the reuse of specimens and information flow among levels of investigation.

We argue that only DNA-based techniques provide sufficient throughput and resolution to enable microbial community surveys at the required scale. The popular methods vary in the information provided, throughput, and cost. None of the methods can address all points listed above on its own, but the comparison of their features suggests how they can be applied jointly.

Diagnostic PCR - a test of whether a marker region of interest, usually a portion of the 16S rRNA gene for a strain or clade of microorganisms, can be amplified from and is thus present in a sample - is the cheapest and conceptually most straightforward method of symbiosis study. **Combined with Sanger sequencing** of selected PCR products, validating specificity, and providing some phylogenetic information, diagnostic PCRs have been used widely and may remain the most direct way of addressing specific questions in the future, as demonstrated by recent high-profile studies focused on certain microbes in particular host species [110,125,140].

High-throughput sequencing of amplicons of marker genes such as bacterial 16S rRNA provides information on the diversity and relative abundance of sequence variants across samples. Despite known biases and challenges [173], the method is suitable for addressing broad microbial diversity and distribution questions. We have recently demonstrated that insect marker genes can be amplified and characterized simultaneously with bacterial markers [174], providing host identity and parasitoid infection information and, thus, a robust framework for microbiota characterization.

Metagenomics - high-throughput sequencing of the total host and microbial DNA extracted from an insect - is the most comprehensive method, providing detailed information about symbionts' and hosts' phylogenetic relationships and functions. Unfortunately, because of high per-sample cost and analysis complexity, few projects have attempted to address microbiome-related

questions in wild insects using more than a few dozen metagenomic datasets [25,31,175]. An interesting alternative is **target enrichment** or **hybrid capture sequencing**. In this approach, before sequencing, pre-selected phylogenetically or functionally informative genes are enriched from metagenomic libraries [176], substantially reducing the sequencing depth (and cost) needed for the sequence reconstruction. However, the method has not been widely applied in insect symbiosis research [177,178].

It is also helpful to consider how DNA-based methods for insect biodiversity surveys can aid microbiome surveys. The most popular approaches are based on high-throughput sequencing of amplicons of insect marker genes, generally mitochondrial cytochrome oxidase (I), or COI [179]. One of these approaches, metabarcoding, uses DNA extracted from whole multi-species community samples, whether homogenized or used for non-destructive partial lysis [180]. The other approach, high-throughput barcoding [179], generates COI amplicon data for large numbers of individual insects following their brief incubation in a lysis buffer. These COI amplicon-based approaches could serve as a helpful starting point in microbiome characterization, providing information on the identity of insects but also informing about the presence of certain microbes. Specifically, barcoding primers are known to amplify COI sequences of alphaproteobacterial symbionts *Wolbachia* and *Rickettsia* in addition to insect genes [181]. Our preliminary analyses suggest that these COI data enable detection and improve phylogenetic resolution relative to 16S rRNA, at least for *Wolbachia* [182]. Detection reliability likely varies depending on the protocols used - DNA extraction strategy, primers, sequencing platform, and depth - and needs to be validated. Nevertheless, mining COI amplicon datasets generated to study insect diversity could provide a window into microbiome patterns extremely cost-effectively.

While interpreting *Wolbachia* genotype information derived from metabarcoding data for multi-species community samples can be tricky, barcoding data for individual insects are more straightforward to interpret. Among the challenges is the scale of the current barcoding projects, sometimes encompassing tens or hundreds of thousands of specimens [183,184]. Besides *Wolbachia* infection information, such extensive collections of pre-barcoded insects could then serve as a material for more complete microbiota characterization. We have recently verified that the non-destructive HotShot treatment, used for obtaining DNA as a part of a cost-effective barcoding workflow [185], does not significantly alter the reconstructed microbial community profiles [186]. Then, we can plausibly use barcoding-derived species IDs to select specimens for processing using more comprehensive methods such as marker gene amplicon sequencing. That way, we can maximize the taxonomic diversity of processed samples while avoiding the oversampling of abundant species and correct for *Wolbachia* infections as a potentially important variable in determining overall microbiota composition. Similarly, marker gene amplicon sequencing data could plausibly be used to select samples for target enrichment sequencing or metagenomics.

A comparison of these methods' features, including throughput, the information provided, and per-sample cost, suggests how methods could be combined into an effective workflow for microbiome surveys at population or community levels and integrated into broader biodiversity surveys (Fig. 4). We can use insects from broad sampling efforts for high-throughput non-

destructive barcoding, providing information on the taxonomic identity of large numbers of individually tracked specimens [185]. Combining barcodes and information on alphaproteobacterial symbiont infections can provide a comprehensive picture of infections with some of the most significant microbes. Simultaneously, it can guide the selection of specimens for microbial marker gene amplicon sequencing, providing a broad picture of microbial genotype and clade distribution across populations and species. These data will likely pinpoint host-symbiont associations that are of particular interest because of their distribution or putative effects on hosts. They could guide the selection of specimens for target enrichment sequencing - providing a high phylogenetic resolution that enables the detection of patterns such as the recent or ongoing horizontal transmission of symbionts across species. Finally, a subset of available metagenomic libraries could be sequenced to high coverage without the enrichment step, enabling the full reconstruction of genomic features and functions. Overall, such a multi-step workflow could start with a broad insect biodiversity characterization, reconstruction of microbiome-related processes and patterns within selected clades and species, and then focus on the biology of selected microbes.

More microbiome-surveying tools may become available soon, thanks to rapid technological progress. Improved reagents and protocols and laboratory automation simplify high-throughput sample processing. The orders-of-magnitude decrease in the cost of 1 Mb of sequence data since the turn of the century and the emergence and rapid improvement of long-read sequencing technologies have opened up new opportunities [187]. The speed and capacity of computational resources have improved in parallel. Arguably, we are no longer limited by technology when addressing extremely broad questions about host-microbiome interactions using DNA-based and computational techniques. Instead, imagination and the availability of highly trained personnel might often be limiting factors. Considering whether the developments in robotics and artificial intelligence [188] might help address these gaps is intriguing. They could help us take advantage of further improvements in the technologies listed above and completely new opportunities. For example, one could wonder to what extent the reconstruction of microbial infection status could be made visually - based on high-throughput analysis of photographs of live insects - alongside their identification [189].
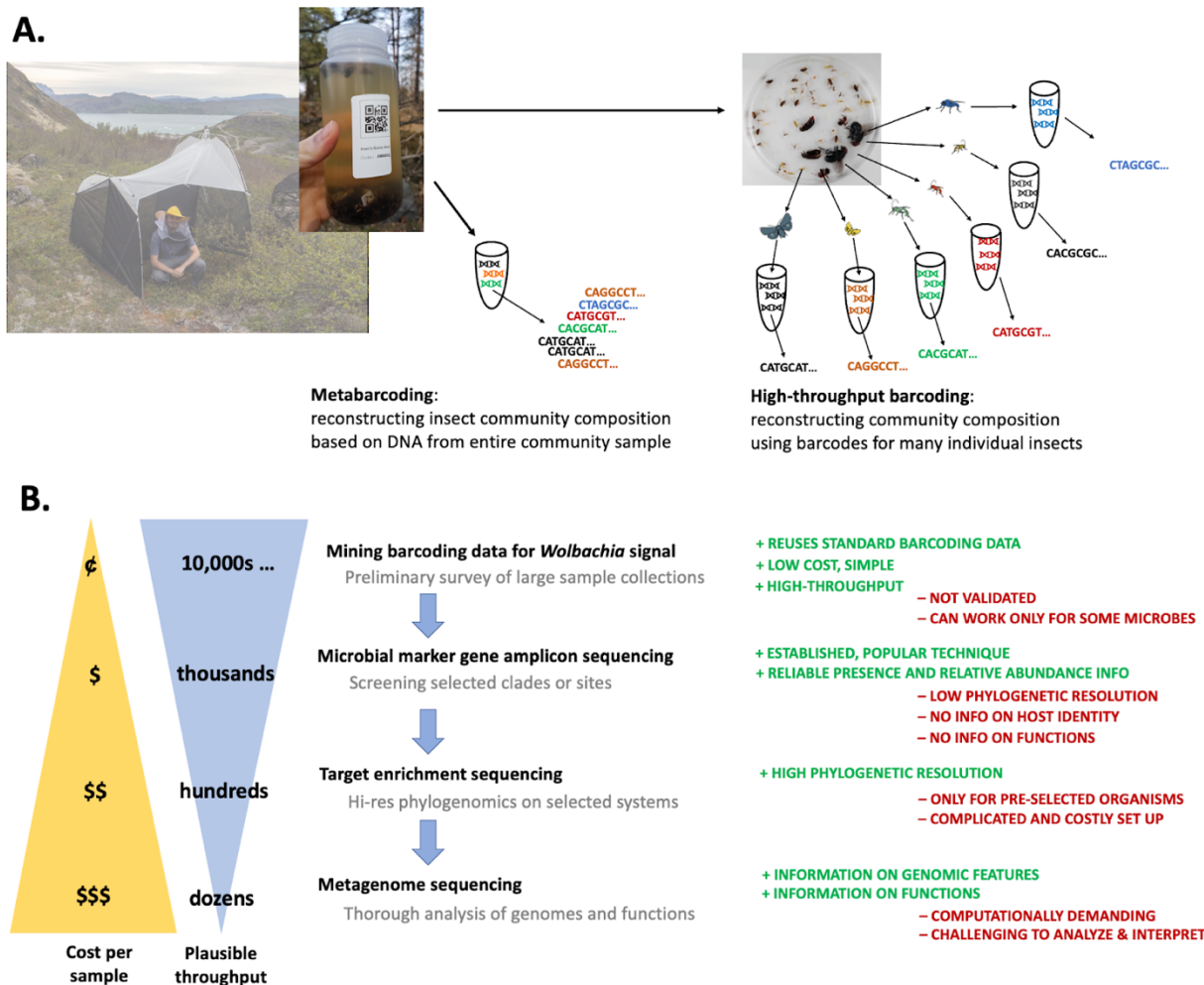
Figure 4. Alternative approaches to the characterization of communities of insects and their associated microbes. A. The primary DNA-based approaches to insect community characterization rely on reconstructing insect marker gene sequences, either from bulk multi-species samples (metabarcoding) or for large numbers of individual insects (barcoding). **B.** The approaches to microbiome characterization in collections of wild insects vary considerably in plausible throughput and per-sample cost. The comparison of their strengths and limitations suggests how they can be applied and combined into a workflow that combines breadth and deep insights into selected symbioses.

Conclusions: insect biodiversity researchers must consider microbiota

Microbial symbionts are a critical component of insect biology: we need to know microbiota to understand insects fully. Symbionts have been critical in the evolution of insect nutrition and present-day nutritional biology, highly relevant in many species of agricultural and medical significance. Symbionts' effects on insect reproduction are a potent means of affecting host population structure and processes such as speciation. Their diverse effects on insect life history traits, interactions with other species, and susceptibility to abiotic factors known to strongly

influence the composition of communities can shape the insects' ecological niche. The symbionts' ability to transmit effectively among insect generations and across lines and species can make them critical agents of rapid response and adaptation to environmental and biotic challenges and opportunities. These diverse effects can be reflected across insects' critical traits: abilities to exploit novel food sources, resist natural enemies, or vector diseases of other animals or plants. They can help insects resist various pressures of the Anthropocene era, including heat, drought, invasive species, and pollution with toxic chemicals.

Understanding insect microbiota is thus essential from both basic and applied perspectives. Characterizing and monitoring microbiota will help us understand changing natural ecosystems during times of global biodiversity crisis and may offer novel opportunities for species conservation. It will be essential for managing insects in agricultural systems as we try to feed the growing global human population under changing environmental conditions while reducing the applications of toxic chemicals and other negative environmental impacts. It will also help control diseases vectored by insects - those directly affecting humans, like dengue and related viruses, and those attacking other animals and plants that matter to ecosystem functions and the human economy.

Considering the microbial symbionts importance, the opportunities for insect microbiota characterization provided by increasing biodiversity surveys seem like an opportunity that is too good to miss. By mining the growing number of insect biodiversity datasets, we could obtain information on some key microbial players in insect biology. High-throughput barcoding datasets and collections could serve as an ideal starting point for characterizing microbial transmission across interacting species that form natural communities. Including additional DNA-based tools in a step-wise manner could provide both a broad perspective and deep insights into the most promising insect-microbe associations.

We are confident that implementing such routines into insect biodiversity monitoring attempts will provide new valuable information about the basic biology of the planet's most diverse animal clade. However, we hope it will also indicate how they could be effectively monitored, managed, and protected in our rapidly changing world.

Acknowledgments

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