Evolutionary Ecology
https://doi.org/10.1007/s10682-022-10197-2

ORIGINAL PAPER

Beyond canonical models: why a broader understanding of Diptera-microbiota interactions is essential for vector-borne disease control

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Received: 5 November 2021 / Accepted: 14 June 2022

Abstract

Vector-borne diseases constitute a major global public health threat. The most significant arthropod disease vectors are predominantly comprised of members of the insect order Diptera (true flies), which have long been the focus of research into host–pathogen dynamics. Recent studies have revealed the underappreciated diversity and function of dipteran-associated gut microbial communities, with important implications for dipteran physiology, ecology, and pathogen transmission. However, the effective parameterization of these aspects into epidemiological models will require a comprehensive study of microbe-dipteran interactions across vectors and related species. Here, we synthesize recent research into microbial communities associated with major families of dipteran vectors and highlight the importance of development and expansion of experimentally tractable models across Diptera towards understanding the functional roles of the gut microbiota in modulating disease transmission. We then posit why further study of these and other dipteran insects is not only essential to a comprehensive understanding of how to integrate vector-microbiota interactions into existing epidemiological frameworks, but our understanding of the ecology and evolution of animal-microbe symbiosis more broadly.

Keywords Gut microbiota · Vectorial capacity · Symbiosis · Insects · Diptera

Introduction

The insect order Diptera (true flies) represents the most significant biological vectors of pathogens on Earth. Indeed, members of this group are essential to transmission of the causative agents of numerous human diseases, including malaria, West Nile fever, dengue, African sleeping sickness, leishmaniasis, and river blindness. Most dipteran

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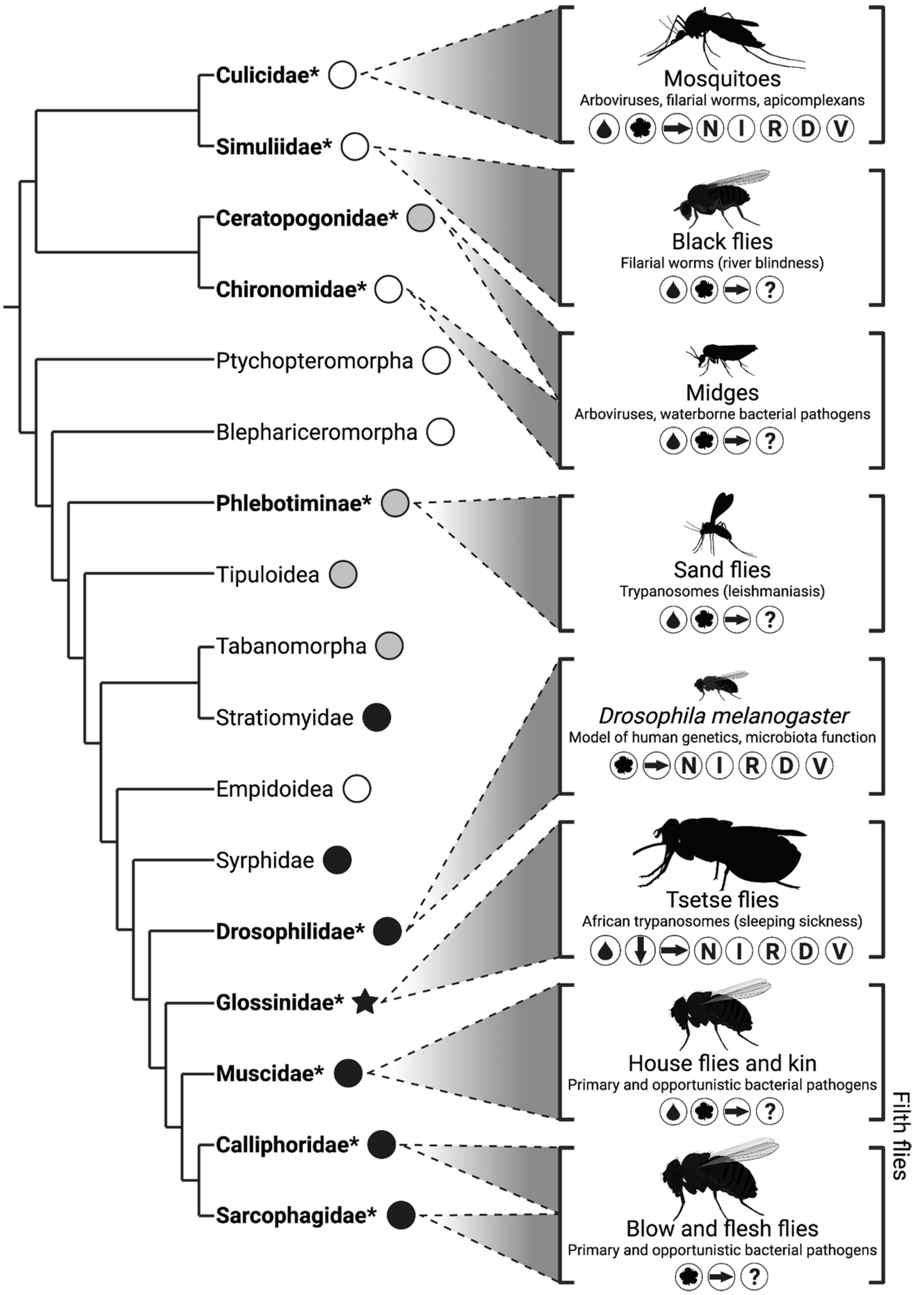
Fig. 1 (*Left*) Phylogenetic overview of larval life history across the insect order Diptera. Circles indicate ▶ whether a clade contains mostly or exclusively aquatic species (white), peripherally aquatic or a mixture of aquatic and terrestrial species (grey), or mostly terrestrial species (black). Clades marked with a black star are viviparous and bear live offspring, while clades marked with an asterisk (*) are addressed in this review. Cladogram modified from Dobson (2013). (*Right*) Overview of the major groups of dipteran disease vectors of focus in this review. Information regarding each group's adult life history, primary vectored pathogens, and associations with non-pathogenic microbes is provided. Groups marked with both a droplet and flower icon contain hematophagous and non-biting species, while groups marked with only a droplet or flower contain only obligately hematophagous or non-biting species, respectively. Icons with horizontal arrows indicate groups for which microbiota are known to be acquired horizontally from the environment, while icons with vertical arrows indicate groups with highly specific associations with bacterial endosymbionts that are vertically transmitted from mother to offspring. Icons containing letters indicate groups with known functions for microbiota in host physiology (N = nutrition; I = immunity; R = reproduction; D = growth and development; V = vector competence). Question marks indicate groups for which microbiota function is poorly elucidated. All groups contain species that are known to be naturally infected by the intracellular bacterium *Wolbachia*. Figure created with BioRender.com

vectors acquire pathogens when they take a blood meal from an infected host (termed 'hematophagy'). Ingested pathogens must thereafter cross the midgut epithelium, replicate, and travel through the hemocoel (insect body cavity) to the salivary glands, from where they will be delivered to a new, susceptible host when they take their next blood meal (Hardy et al. 1983; Abraham and Jacobs-Lorena 2004). Each step in this process presents barriers to invading pathogens. In the midgut, these include the peritrophic matrix, proteolytic enzymes and toxic products associated with blood meal digestion, and immune defense molecules. They also include resident gut microbiota, which as described herein are well-known to modulate host interactions with invading pathogens, through direct and indirect mechanisms. As such, most functional studies in dipteran disease vectors have focused on understanding the mechanisms by which gut microbiota influence the competence of different species to vector human pathogens (*i.e.*, 'vector competence'). However, while a particular species may serve as a competent vector for a given pathogen, pathogen transmission dynamics at the population level may be influenced by several intrinsic and extrinsic factors. The potential for a vector population to transmit a pathogen within a susceptible host population is referred to as 'vectorial capacity' (Garrett-Jones 1964; Garrett-Jones and Grab 1964). Multiple elements of vector biology govern vectorial capacity, including population


Culicidae*
Simuliidae*
Ceratopogonidae*
Chironomidae*
Ptychopteromorpha
Blephariceromorpha
Phlebotiminae*
Tipuloidea
Tabanomorpha
Stratiomyidae
Empidoidea
Syrphidae
Drosophilidae*
Glossinidae*
Muscidae*
Calliphoridae*
Sarcophagidae*
Mosquitoes
Arboviruses, filarial worms, apicomplexans
N I R D V
Black flies
Filarial worms (river blindness)
?
Midges
Arboviruses, waterborne bacterial pathogens
?
Sand flies
Trypanosomes (leishmaniasis)
?
Drosophila melanogaster
Model of human genetics, microbiota function
N I R D V
Tsetse flies
African trypanosomes (sleeping sickness)
N I R D V
House flies and kin
Primary and opportunistic bacterial pathogens
?
Blow and flesh flies
Primary and opportunistic bacterial pathogens
?
Filth flies

density, average lifespan, host feeding preferences, and vector competence. In this way, factors that influence basic physiological processes of vectors, such as their survival, reproduction, growth, and development, have the potential to impact the spread of the pathogens they transmit.

Here, we synthesize recent research into the diversity and function of gut microbiota in hosts spanning the phylogenetic breadth of the order Diptera, with an emphasis on the impacts of gut microbiota on host physiology in both canonical and non-canonical human disease vectors (i.e., those transmitting human pathogens that require the vector to develop from one stage to another *versus* those that transmit human pathogens without amplification or development) (Fig. 1). We then posit why further study of these and other dipteran insects, including non-vector species, is not only essential to a comprehensive understanding of how to integrate vector-microbiota interactions with existing epidemiological frameworks, but our understanding of the ecology and evolution of animal-microbe symbiosis more broadly.

Current knowledge of gut microbial diversity and function in canonical dipteran disease vectors

Mosquitoes (Diptera: Culicidae)

Mosquitoes account for over 300 million annual cases of vector-borne disease worldwide, transmitting the causative agents of human malaria (*i.e.*, *Plasmodium* parasites of the phylum Apicomplexa), various arboviruses including dengue and Zika virus, as well as parasitic nematodes causing filariasis (Franklinos et al. 2019). Considering continued global change, the geographic range of many vector mosquito species is expected to increase, and with it the regions and populations at risk for contracting mosquito-vectored infectious diseases (Franklinos et al. 2019). The efficiency of mosquito disease transmission is owed in large part to the varied habitats in which they can successfully reproduce and their ability to easily adapt to urban landscapes in response to global changes in human land use patterns (Franklinos et al. 2019).

Mosquitoes acquire their gut microbiota at several stages throughout their life cycle. As larvae, mosquitoes inhabit a wide variety of aquatic habitats where they filter feed on detritus and other organic matter, including bacteria and other microorganisms present in the water column (Coon et al. 2014, 2016a; Gimonneau et al. 2014; Wang et al. 2018; Bascuñán et al. 2018). Deep sequencing of bacterial 16S rRNA gene and fungal ITS amplicons shows that the gut microbiota within larvae of *Aedes aegypti* and other vector species of mosquitoes is dominated by a relatively small number of unique taxa (~200) that vary substantially as a function of collection site (Coon et al. 2016a; Dickson et al. 2017; Tawidian et al. 2021). The bacteria present in larvae also nearly completely overlap with the bacteria present in their aquatic environment, although community diversity is lower, and the abundance of specific community members differs (Coon et al. 2014, 2016a; Gimonneau et al. 2014; Bascuñán et al. 2018; Wang et al. 2018). Controlled experiments further indicate that while bacteria reside on the surface of egg masses females lay, larvae that hatch from surface-sterilized eggs in sterile water contain no bacteria in their digestive tract (Coon et al. 2014). Altogether, these data strongly support that mosquito larvae acquire their gut microbiota from the water in which they feed.

Larvae molt through four instars before undergoing metamorphosis to form pupae that float on the surface of the aquatic habitat (Clements 1992). Pupae do not feed, and no new microorganisms are introduced into the gut at this stage. Adults emerge from the pupal stage and persist in terrestrial habitats where both sexes feed on plant nectar and other sugar sources (Clements 1992). Adult females of most species also feed on vertebrate blood in order to produce eggs for the next generation (Clements 1992), which as mentioned previously is how they acquire and transmit disease-causing organisms. Newly emerged adults are generally thought to contain very few bacteria (Moll et al. 2001; Dickson et al. 2017). This is because larvae void the contents of their gut prior to pupation, and any remaining bacteria are sequestered with the degenerating larval gut during metamorphosis to form a meconium that is egested by adults immediately after emergence (Moll et al. 2001). Culture-based studies support a drastic reduction in bacterial counts just prior to and following metamorphosis (Moll et al. 2001). However, deep sequencing of communities from adults allowed to emerge from pupae maintained under sterile conditions also indicates that a subset of the bacteria present in larvae can persist to the adult stage (termed ‘transstadial transmission’) (Lindh et al. 2008; Coon et al. 2014, 2016a; Gimonneau et al. 2014; Bascuñán et al. 2018; Wang et al. 2018). Newly emerged adults further immediately imbibe water from their larval habitat, which reseeds the gut with environmental microbes. However, while the relative contributions of transstadial transmission and environmental acquisition in shaping the composition of gut microbiota in adult mosquitoes are unknown, bacterial diversity in adults is consistently much lower than in larvae (<20 taxa) and varies tremendously across species, among individuals, and as a function of feeding status (Kent et al. 1996; Lindh et al. 2008; Wang et al. 2011; Álvarez-Pérez et al. 2012; Coon et al. 2014; Gimonneau et al. 2014; Duguma et al. 2015).

Studies of microbiota function in mosquitoes have been greatly facilitated by the recent development of sterilization techniques for the generation of microbe-free (‘axenic’) and selectively colonized (‘gnotobiotic’) mosquito larvae and adults (Coon et al. 2014), as well as the “decolonization” of conventionally reared individuals via antibiotics or other methods (Romoli et al. 2021). Results of these studies collectively indicate that mosquito gut microbiota play profound roles in their growth and development, survival, and reproduction (Xiao et al. 2017; Barnard et al. 2019; Chabanol et al. 2020; Romoli et al. 2021). For example, vector species of mosquitoes within the genera *Aedes, Anopheles,* and *Culex* have been reported to rely on microbe-induced gut hypoxia as a signal for development (Coon et al. 2014, 2016a; Valzania et al. 2018b). Recent research shows that members of the mosquito gut microbiota induce gut hypoxia, larval growth, and molting by specifically provisioning larvae with riboflavin, while other microbially-derived B vitamins and exogenous metabolites, including folate, pyridoxine, thiamine, and dietary sterols, play important roles in shaping larval growth and pupation rates (Wang et al. 2021). The gut microbiota in adult mosquitoes has similarly been shown to aid in blood meal digestion, nutrient assimilation, and egg production in *Aedes aegypti* and related mosquito species (Gaio et al. 2011). Gut microbiota can also affect the competence of individual mosquitoes to transmit pathogens to humans, via activation of mosquito immune responses, resource competition, the production of anti-pathogen molecules, or induction of proper formation of the peritrophic matrix in the adult midgut post-blood-feeding, which serves as the first barrier to pathogen entry (exhaustively reviewed in Dennison et al. 2014; Hegde et al. 2015; Romoli and Gendrin 2018; Caragata et al. 2019; Scolari et al. 2019; Souza-Neto et al. 2019; Gao et al. 2020).

While not considered a member of the gut microbiota, several mosquito species are also often infected by the intracellular bacterium *Wolbachia*, which is maternally transmitted by many arthropods, including other hematophagous insects (see below) (Weinert et al. 2015). *Wolbachia* bacteria are known to contribute to several phenotypes of interest in mosquitoes, including ‘cytoplasmic incompatibility’ (CI), which, over time, can lead to a decrease in the reproductive potential of adult females and ultimately the local elimination or eradication of a mosquito population (Bourtzis 2008). Certain

Wolbachia strains have also been demonstrated to suppress propagation and transmission of dengue virus and malaria parasites in *Wolbachia*-infected mosquitoes (as reviewed in Bourtzis et al. 2014; Flores and O'Neill 2018; Caragata et al. 2019), while also aiding mosquito hosts via the provisioning of certain essential cofactors and vitamins (as reviewed in Kaur et al. 2021). Interactions between *Wolbachia* and members of the native mosquito gut microbiota are largely understudied, although recent studies support a role for such interactions in shaping the establishment and persistence of pathogen-blocking *Wolbachia* strains in mosquito hosts (Hughes et al. 2014). *Wolbachia*-gut microbiota interactions may also act in synergy to enhance immune priming phenotypes in *Ae. aegypti* (Ye et al. 2013) or antagonistically to reduce the relative abundance of beneficial gut microbes to the detriment of the mosquito, although consistent patterns in such antagonistic and/or synergistic interactions between specific *Wolbachia* and gut microbial strains have not been conclusively identified (Chen et al. 2016; Audsley et al. 2017; Straub et al. 2020).

Black flies (Diptera: Simuliidae)

Black flies of the genus *Simulium* are vectors of the parasitic worm *Onchocerca volvulus*, the causative agent of onchocerciasis (river blindness). Considered a neglected tropical disease, the at-risk population for onchocerciasis is primarily located in sub-Saharan Africa, but cases have also been reported in areas of Latin America (Burki 2021). Black flies develop as larvae in oxygenated water sources, with terrestrial adults usually being associated with slow-moving streams, creeks, or rivers where the immature stages develop. Like mosquitoes, most black fly larvae are filter feeders, with the larvae feeding on detritus and other organic matter in the water as it flows by. Pupae do not feed, and instead remain attached to vegetation or other stationary objects before floating to the surface for adult emergence. Adults of both sexes feed on nectar and other sugar sources, while adult females of most species also must blood feed to produce eggs.

Given the striking similarities in their life histories, it is likely that patterns of gut microbiota acquisition and persistence in black flies mirror those of mosquitoes, although to date almost nothing is known about the black fly gut microbiota, let alone what interactions or impacts particular microbial species or assemblages may have on the successful establishment or transmission of *O. volvulus.* A single study recently used culture-based methods to characterize bacterial diversity in newly emerged *Simulium tani* adult flies from field-collected pupae (Lee et al. 2021a). Sex- and species-specific patterns in bacterial diversity within *S. innoxium* larvae and newly emerged *S. inooxium, S. dixiense, S. krebsorum,* and *S. slossonae* adults from field-collected pupae have also been investigated using culture-independent approaches via high throughput 16S rRNA gene amplicon sequencing (Tang et al. 2012). These studies collectively suggest that adult black flies harbor a relatively simple gut bacterial community dominated by ~ 20 species that are commonly detected in the freshwater habitats where black fly larvae develop (Tang et al. 2012; Lee et al. 2021a). Bacterial diversity in adult females is marginally lower than that in males, although there are dramatic differences in the presence and abundance of specific community members, including taxa within the bacterial families *Enterobacteriaceae*, *Sphingomonadaceae*, *Pseudomonadaceae*, and *Moraxellaceae*, which are known to proliferate in adult mosquitoes (Tang et al. 2012; Duguma et al. 2015; Muturi et al. 2019). Similar taxa are also detected in the guts of larvae derived from the same collection sites. This, combined with the fact that patterns in bacterial community composition do not seem to correlate with host phylogeny, suggests that black flies likely largely acquire their gut microbiota from the environment as larvae, with a subset persisting to the adult stage as has previously been demonstrated in mosquitoes.

Finally, while *Wolbachia* is commonly detected in many dipteran insects including mosquitoes, evidence for natural infection in *Simulium* spp. and the potential to harness *Wolbachia* for black fly control is comparatively lacking, although *Wolbachia* is regularly detected in the *O. volvulus* filarial worms they vector, and depletion of *Wolbachia* in *O. volvulus* is well-recognized as a treatment for onchocerciasis (André et al. 2002). To date, only a single instance of the presence of seasonally stable *Wolbachia* infection has been documented in *Simulium* spp. (Woodford et al. 2018). The same study also reported host species-specific impacts of *Wolbachia* infection on the ability of adult female flies to transmit the avian malaria parasite, *Leucocytozoan* (Woodford et al. 2018). Whether similar impacts are observed in the context of *O. volvulus* transmission is currently unknown. The potential of *Wolbachia* bacteria and members of the gut microbiota to modulate black fly fitness is also poorly understood, owing to the absence of controlled studies in the laboratory. While large-scale rearing of black flies has been reported previously (Kiszewski et al. 1988), scaling of these methods to allow for microbiota manipulation in individual flies will be essential for expanding our understanding of gut microbiota assembly and function in this group of dipteran disease vectors.

Sand flies (Diptera: Psychodidae)

Hematophagous sand flies of the (sub)family Phlebotominae serve as the primary vectors of *Leishmania*, a genus of trypanosome parasites implicated in 20,000–40,000 deaths annually (Lainson and Shaw 1968; Alvar et al. 2012). Unlike mosquitoes and blackflies, sand flies do not breed in water and larvae instead develop in warm, humid terrestrial habitats containing decaying organic matter (e.g., rock crevices, rodent burrows, tree holes, caves). Terrestrial adults of both sexes, like mosquitoes and black flies, emerge from the non-feeding pupal stage to feed on plant juices and sugary excretions while only adult females must blood feed to produce eggs (Volf et al. 2002).

Interest in sand fly gut microbiota has increased recently given results showing that it plays an essential role in sand fly vector competence for *Leishmania* parasites (Louradour et al. 2017; Dey et al. 2018). Like mosquitoes and black flies, sand flies acquire their gut microbiota as larvae by feeding on the organic matter present in the humid soil in which they develop. A subset of this larval gut microbiota is subsequently transstadially transmitted to the adult stage (Peterkova-Koci et al. 2012; Campolina et al. 2020) and thereafter may be modulated in response to sugar and/or blood feeding, the latter of which has been documented to have marked impacts on sand fly-associated gut microbial communities by inducing the proliferation of specific taxa known to tolerate the oxidative stress following blood meal digestion in other facultatively hematophagous dipteran species (Dillon et al. 1996; Volf et al. 2002; Sant'Anna et al. 2014; Maleki-Ravasan et al. 2015; Monteiro et al. 2016; Pires et al. 2017). Several studies have demonstrated the ability of related taxa, including *Serratia marcescens*, to interfere with *Leishmania* development inside the sand fly vector (Grimont et al. 1979; Moraes et al. 2008, 2009; Hassan 2014; Sant'Anna et al. 2014) via priming of the host immune system, as observed in *Anopheles gambiae* mosquitoes infected with *Plasmodium* (Boissière et al. 2012). Antibiotic treatment has also been demonstrated to increase the susceptibility of certain sand fly host species to *Leishmania* infection (Hassan 2014); similar to studies in other dipteran disease vectors including mosquitoes, impacts of antibiotic treatment on vector competence can vary tremendously as a function of host and parasite species or strain, with antibiotic treatment of sandflies in some studies actually inhibiting parasite growth and differentiation into the infectious form (termed 'metacyclogenesis') (Kelly et al. 2017; Louradour et al. 2017). Research into impacts of gut microbiota on aspects of sand fly physiology outside of vector competence (e.g., growth and development) is comparatively lacking, although initial evidence suggests that terrestrial sand fly larvae can be successfully reared to the adult stage under axenic conditions with impacts only on growth and survival rates (Peterkova-Koci et al. 2012). These results contrast sharply with aquatic mosquito larvae, which require a living gut microbiota to develop past the first instar under normal environmental conditions (Coon et al. 2014, 2016b, 2020; Valzania et al. 2018a, b; Wang et al. 2021).

Limited research exists into the prevalence and function of endosymbiotic bacteria in sand flies, although surveys have reported the presence of both *Wolbachia* and *Spiroplasma*, the latter of which is a well-characterized vertically transmitted endosymbiont of *Drosophila* spp. (Diptera: Drosophilidae) (Ono et al. 2001; Parvizi et al. 2013; de Oliveira et al. 2015; Karatepe et al. 2018). Infection rates of these endosymbionts among and between sand fly species and populations appear to be highly variable in the field, and the impacts of infection on sand fly reproduction or other fitness phenotypes have not been characterized. Recent transinfection of *Lutzomyia* cell lines with different *Wolbachia* strains shows that stable microbiota manipulation may be possible in sand flies, although cell line susceptibility to *Leishmania infantum* was not affected by transinfection status (da Silva Gonçalves et al. 2019). The expansion of methods to manipulate sand fly-associated microbial communities in vivo in the laboratory will be essential to identify candidate gut microbes and/or endosymbionts that can be harnessed for sand fly and *Leishmania* control. A comprehensive understanding of how sand fly-associated microbial communities vary in time and space within and between different Phlebotomine sand fly species, as well as the characterization of microbial communities to include genomic information beyond just the 16S rRNA gene, will also be essential for predicting the success of deployment of such candidates in the field.

Tsetse flies (Diptera: Glossinidae)

Tsetse flies are vectors of protozoan parasites within the genus *Trypanosoma*, which are the causative agents of trypanosomiasis (sleeping sickness). The population at risk encompasses 36 countries in sub-Saharan Africa and 65 million people (Simarro et al. 2012). Tsetse flies are unique among dipteran disease vectors in that they are viviparous and bear live offspring. Adult females produce a single egg at a time, which passes into the uterus where it is fertilized and eventually develops through three larval instars before leaving the mother, pupating in the surrounding soil, and emerging as an adult. Larval stages develop by feeding on a milk-like substance synthesized by the mother's milk gland, while adults of both sexes feed exclusively on vertebrate blood, from which digested proteins are used to synthesize and store fat for flight, mating, and milk production by females.

Owing to their unique life history, most research into tsetse fly-associated microbiota has focused on one of two bacterial symbionts: the obligate symbiont *Wigglesworthia glossinidia*, which is found in all tsetse flies, and the facultative symbiont *Sodalis glossinidius*, which has a variable distribution across fly species and populations (Dennis et al. 2014; Tagueu et al.

2018). Both symbionts are largely housed intracellularly within specialized host organs (the bacteriome and milk gland), which facilitates their vertical transmission to developing offspring (Ma and Denlinger 1974; Pais et al. 2008), while *S. glossinidius*, unlike *W. glossinidia*, is also sometimes found extracellularly in the host midgut lumen (Cheng and Aksoy 1999; Balmand et al. 2013). *Wigglesworthia glossinidia* plays an essential role in the provisioning of nutrients and B vitamins lacking in the blood adults consume and is therefore also essential for larval development within adult females and maturation of the developing offspring's immune systems (as reviewed in Rio et al. 2016). In contrast, no specific functional contributions of *S. glossinidius* toward tsetse fly biology have been identified, although recent studies suggest both *W. glossinidia* and *S. glossinidius* can impact tsetse fly vector competence for trypanosomes (Medina Munoz et al. 2021). For example, *W. glossinidia* symbionts are known to provision folate (vitamin B9) to both their tsetse fly hosts and associated trypanosomes, both of which are unable to synthesize this compound de novo in the absence of symbionts. In this way, the presence and abundance of *W. glossinidia* has the potential to enhance the permissiveness of different tsetse fly species to trypanosome establishment (Medina Munoz et al. 2021). In contrast, expression of *S. glossinidius* genes involved in chitin metabolism may facilitate trypanosome establishment by damaging the tsetse fly host's peritrophic matrix (Medina Munoz et al. 2021).

Much less well characterized is the diversity and abundance of extracellular microbes present in the tsetse fly gut lumen, due to most deep sequencing efforts being conducted using libraries generated from whole-body fly homogenates and therefore dominated by *W. glossinidia* and *S. glossinidius* reads (Gaithuma et al. 2020). However, despite these difficulties, members of the bacterial genera *Bacillus*, *Pseudomonas*, *Corynebacterium*, *Paracoccus,* and *Acinetobacter* have been detected at low relative abundances in sequencing data (Gaithuma et al. 2020), and culture-dependent approaches have proven successful in isolating bacteria from the genera *Bacillus, Acinetobacter, Mesorhizobium, Paracoccus, Microbacterium, Micrococcus, Arthrobacter, Corynebacterium, Curtobacterium, Vagococcus,* and *Dietzia* (Malele et al. 2013). To date, only two studies have conducted deep sequencing using libraries prepared with DNA from midguts of dissected tsetse flies that had previously taken a blood meal and were either infected or uninfected with trypanosome parasites (Griffith et al. 2018; Ngambia Freitas et al. 2021). In addition to detected higher relative abundances of *S. glossinidius* in the guts of infected tsetse flies, these studies also identified distinct, low-density communities comprised of <10 environmentally acquired bacteria that differed between individuals depending on infection status and the location from which they were collected (Griffith et al. 2018; Ngambia Freitas et al. 2021). The mechanisms that underlie colonization of the tsetse fly midgut by environmental bacteria requires further investigation. However, the dynamics of this process are presumably different from that which occurs in other well-studied dipteran disease vectors. For example, free-living mosquito larvae acquire nutrients directly from the aquatic habitats in which they develop, and therefore house a complex gut microbiota consisting of a subset of the equally complex microbial communities inhabiting the surrounding environment (Coon et al. 2014, 2016a; Gimonneau et al. 2014; Bascuñán et al. 2018; Wang et al. 2018). In contrast, tsetse fly larvae develop entirely within the confines of their mothers' uteri, which are devoid of environmental microbes resulting in larvae being exposed exclusively to maternally transmitted *Wigglesworthia* and *Sodalis* symbionts, and in some cases *Wolbachia* (Rio et al. 2006). In this way, tsetse flies only acquire food from the environment during the adult stage of their life cycle, via repeated interactions with vertebrate hosts during blood feeding activities (Gaithuma et al. 2020) and potentially during mating, as has been observed in mosquitoes (Damiani et al. 2008). That tsetse flies are obligately hematophagous also presents more opportunities for blood feeding behaviors to shape tsetse fly-associated gut microbial communities over time, either by the introduction of vertebrate host-specific microbiota or the repeated bottlenecking of communities in response to the oxidative stress associated with blood meal digestion (Clausen et al. 1998). Further experimental studies are required to decipher the mechanisms that underlie colonization of the tsetse fly gut by environmental bacteria and the functional roles of individual bacteria and assemblages on tsetse fly vector competence and physiology, including the influence of resident endosymbionts like *W. glossinidia* and *S. glossinidius*.

Current knowledge in non-canonical dipteran disease vectors

Filth flies (Diptera: Muscidae, Calliphoridae, Sarcophagidae)

Filth flies within the dipteran families Muscidae, Calliphoridae, and Sarcophagidae have long been implicated as potential vectors for various bacterial pathogens in humans and other animals, including—but not limited to—enteropathogenic *Escherichia coli, Helicobacter pylori,* and *Salmonella* spp. (Ostrolenk and Welch 1942; Greenberg 1965; Bidawid et al. 1978; Forsey and Darougar 1981; Haseyama et al. 2015; Khamesipour et al. 2018; Junquiera et al. 2017). These include hematophagous flies in the genera *Stomoxys* (stable flies) and *Haematobia* (horn flies) as well as non-biting species within the genera *Musca* (house and face flies), *Chrysomya* (blow flies), and *Sarcophaga* (flesh flies), all of which complete their entire lifecycle within habitations of humans and domestic animals (Moon 2019). Owing to this lifestyle, there are recurring opportunities for such flies to acquire and transmit environmental pathogens. Adult female filth flies deposit their eggs on feces, animal manure, carrion, or some other decomposing organic substance, which is rich in undigested carbohydrates, proteins and other nutrients and supports development of larvae to the adult stage (Hansens 1963; Meyer and Petersen 1983; Hanski 1987). Both adult male and female flies opportunistically ingest nutrient-rich manure or carrion (West 1951; Hanski 1987; Sasaki et al. 2000). Female flies are also anautogenous and can use manure/carrion, animal secretions, and other bodily fluids (e.g., blood in the case of hematophagous species) to provide the necessary proteins for egg development (Moon 2019). Adult flies are ideal mechanical vectors as their bodies are covered in small hair-like projections, which collect debris from their microbe-rich surroundings and can lead to dissemination of external microbes (Roberts et al. 2013). Mature flies may also acquire environmental pathogens through feeding and/or transstadial transmission from larval stages, which can then be spread via regurgitation or defecation (Butler et al. 1977; Sasaki et al. 2000; Rochon et al. 2005; Mcgaughey and Nayduch 2009; Joyner et al. 2013; Wasala et al. 2013; Zurek and Nayduch 2016; Junqueira et al. 2017). For example, recent high-throughput sequencing studies in swine manure vermicomposting systems exposed to *Musca domestica* larvae have reported rapid reductions ($>75\%$) in the proportion of bacterial reads originating from raw compost and the rapid increase in the proportion of reads originating from *M. domestica* larvae ($\sim 30\%$) over the same time frame (Wang et al. 2017). Similar studies have also been conducted in the black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae), where vermicomposting is linked to the enrichment of larval-associated microbial communities in food waste (Jiang et al. 2019).

The capacity of pathogenic bacteria to colonize and persist in the fly gut is likely greatly influenced by the diversity and abundance of native gut microbiota, which to date is relatively understudied in filth flies when compared to canonical disease vectors. However, while no study has rigorously assessed the factors shaping gut microbiota assembly and diversity across filth fly species, studies do collectively support dominant roles for environment, life stage, and feeding status in shaping fly-associated gut microbial communities, as is well documented in mosquitoes (Zurek et al. 2000; Graczyk et al. 2001; Nayduch et al. 2002; Dillon and Dillon 2004; Rochon et al. 2005; Gupta et al. 2012, 2014; Lemaitre and Miguel-Aliaga 2013; Zurek and Nayduch 2016; Xue et al. 2019; de Jonge et al. 2020). The impact of gut microbiota on (i) the competency of flies to acquire and transmit pathogenic bacteria, and/or (ii) features of their biology that govern vectorial capacity (*e.g.,* growth and development) has also not been comprehensively examined. However, studies do suggest that like sand flies, terrestrial larvae of filth flies can develop into adults under axenic conditions, although pupation and survival rates are often significantly lower than for larvae reared under conventional (non-sterile) conditions and vary as a function of diet (Greenberg 1954; Hollis et al. 1985; Schmidtmann and Martin 1992; Watson et al. 1993; Lysyk et al. 1999; Zurek et al. 2000). Gnotobiotic filth fly larvae monocolonized by different bacterial isolates, like those of mosquitoes and sand flies, also exhibit variable pupation rates under different diet conditions (Greenberg 1954; Hollis et al. 1985; Schmidtmann and Martin 1992; Watson et al. 1993; Lysyk et al. 1999; Zurek et al. 2000; Linenberg et al. 2016; Solomon et al. 2019; McMullen et al. 2020), underscoring the importance of diet-microbe interactions in shaping host fitness. In this way, microbes that promote larval growth and development are likely to increase in their prevalence and abundance in both host populations and their associated environments over time, and this may serve as a previously underappreciated mechanism by which specific microbes, including pathogenic bacteria, may be disseminated.

Both *Wolbachia* and *Spiroplasma* endosymbionts have also been identified from natural populations of a handful of filth fly species (Junqueira et al. 2017), although surveys to date have been relatively limited in scope as compared to other dipteran disease vectors and populations of many medically important filth fly species have yet to be examined. The vast majority of functional studies similarly have focused on one of two subspecies of *Haematobia* (Diptera: Muscidae)–the horn fly, *H. irritans*, and the buffalo fly, *H. irritans exigua*–both of which are known to be competent hosts of *Wolbachia*, including strains that negatively impact fly fitness (Jeyaprakash et al. 2000; Floate et al. 2006; Hornok et al. 2008; Zhang et al. 2009; Palavesam et al. 2012; Torres et al. 2012; Madhav et al. 2020a, b). Thus, future studies–at minimum–must be undertaken to (i) establish whether *Wolbachia* infection is ubiquitous in other filth flies, and (ii) characterize the impact of infection with different *Wolbachia* strains on the biology of diverse host species. A more comprehensive understanding of the diversity and function of microbial communities associated with not only different filth fly species, but their respective breeding environments will also be essential for efforts to manipulate filth fly-associated microbiota for pathogen control, given the potential of both flies and the environment to serve as bacterial transmission reservoirs. Further, the occurrence and abundance of opportunistic and/or potentially pathogenic bacteria in environmental transmission reservoirs is also likely to be greatly impacted by fly abundance and behavior, the former of which is likely to be determined at least in part by the nutritional conditions in different breeding habitats. Different fly and environmental reservoirs may also harbor microbes that belong to clinically relevant taxa but that do not cause disease in humans or other animals. In this way, broader studies comparing larval and adult fitness across a range of nutritional conditions and microbial backgrounds, along with parallel studies to predict or quantify the pathogenicity of any bacterial strains of interest, are strongly needed to fully elucidate pathogen prevalence, abundance, and dispersal in environments where various filth flies persist.

Midges (Diptera: Ceratopogonidae, Chironomidae)

Biting and non-biting midges belonging to the dipteran families Ceratopogonidae and Chironomidae, respectively, have been implicated in the persistence and spread of bacterial pathogens in aquatic environments with subsequent health hazards to humans (Broza and Halpern 2001; Figueras et al. 2011; Halpern and Senderovich 2015; Mee et al. 2017). Adult female biting midges within the genus *Culicoides* can also acquire and transmit viruses such as bluetongue virus, African horsesickness virus, and epizootic hemorrhagic disease virus between horses and ruminants through blood feeding activities (Mellor et al. 2000). Midges are the most abundant insects in freshwater habitats and water supply systems, where detritivorous larvae complete development and provide a food resource for fish, birds, and other invertebrates (Laviad and Halpern 2016). Such environments are well known to regularly experience the addition of anthropogenic pollutants such as fecal waste and domestic sewage, which could be ingested by midge larvae and mobilized through food webs (Laviad-Shitrit et al. 2019; Ding et al. 2021). Indeed, midge egg masses, larvae, and adults are all natural reservoirs for *Vibrio cholerae* and pathogenic *Aeromonas* and *Elizabethkingia* spp., which colonize the gut along with other environmental bacteria during feeding (Broza and Halpern 2001; Figueras et al. 2011; Mee et al. 2017; Ding et al. 2021). The same and related bacterial taxa are also commonly detected in aquatic larvae, terrestrial adults, and/or egg masses of mosquitoes and black flies (Pidiyar et al. 2002; Kämpfer et al. 2011; Tang et al. 2012; Coon et al. 2016a), for which gut microbial diversity is largely shaped by the aquatic environments in which larvae develop.

Studies have only very recently been initiated to improve our understanding of gut microbial diversity and function in midges, with only a few preliminary patterns beginning to emerge. First, like their closest relatives (mosquitoes and black flies), midges appear to acquire their gut microbiota from the aquatic environments in which they develop as larvae (Díaz-Sánchez et al. 2018; Sela et al. 2020; Laviad-Shitri et al. 2021; Möhlmann et al. 2021). Thereafter, gut microbial diversity and the abundance of specific microbial taxa are shaped by life stage and feeding status, with the most dramatic differences being observed between adults of biting *versus* non-biting species and within biting species before *versus* after blood feeding (Díaz-Sánchez et al. 2018; Sela et al. 2020; Laviad-Shitri et al. 2021; Möhlmann et al. 2021). Blood feeding specifically results in the proliferation of bacteria within the families *Enterobactericeae, Pseudomonadaceae*, and *Moraxellaceae* and a decline in overall microbial diversity, as previously mentioned for mosquitoes, black flies, and sand flies (Campbell et al. 2004; Nayduch et al. 2015; Díaz-Sánchez et al. 2018; Möhlmann et al. 2021). Second, like mosquitoes and sand flies, blood meal-associated fluctuations in gut microbiota are also correlated with changes in the expression of genes encoding regulatory components of each of the major conserved immune signaling pathways in biting midges and the upregulation of genes encoding downstream immune effectors such as antimicrobial peptides (Nayduch et al. 2015). Resident gut bacteria may also dampen virus transmission. Antibiotic treatment has been demonstrated to increase infection rates in laboratory populations of *Culicoides nubeculosus* with Schmallenberg virus (Möhlmann et al. 2020). Differences in bacterial community composition have also been observed between naturally occurring *Culicoides* populations with variable competencies to transmit bluetongue virus (Campbell et al. 2004). Finally, preliminary studies indicate that aquatic chironomid larvae require a gut microbiota to complete development from eggs to adults, in a manner similar to mosquitoes but dissimilar to terrestrial larvae of sand and filth flies (Adam Wong, pers. comm.).

Wolbachia endosymbionts have been identified in laboratory strains of *Culicoides sonorensis* and in natural populations of other *Culicoides* spp. (Nakamura et al. 2009; Morag et al. 2012; Mee et al. 2015; Pages et al. 2017; Covey et al. 2020). More recent studies have also demonstrated the ability of at least one *Wolbachia* strain to inhibit bluetongue and epizootic hemorrhagic fever viruses in a *C. sonorensis* cell line (Matthews et al. 2022). However, transinfection experiments to determine if stable *Wolbachia* infections can be established in different *Culicoides* spp. in vivo have yet to be undertaken. Whether naturally occurring infections result in any reproductive and/or virus inhibitory phenotypes that could be used to manipulate natural populations or for disease control is also unknown, despite low-density *Wolbachia* infections being reported in multiple *Culicoides* spp. worldwide. Addressing both questions, along with questions related to the acquisition, persistence, and function of native gut microbiota in the presence or absence of *Wolbachia* infection, will be difficult given the current inability to colonize many *Culicoides* and other midge species in the laboratory.

Insights from a non-vector species: the quintessential model insect, *Drosophila melanogaster* (Diptera: Drosophilidae)

Gut microbial diversity and function has perhaps been most extensively examined in non-vector dipteran species like *D. melanogaster*, which, along with other drosophilid fruit flies, are saprophytic and live in a wide range of terrestrial habitats where larvae hatch from eggs laid on rotting fruits (*i.e.*, a mixture of microbes and plant material) that serve as their primary source of nutrition. Late-stage larvae pupate in the nearby soil before emerging as adults, which feed on the same substrates as larvae, including yeast, bacteria, and other microorganisms present on the surfaces of fruits and leaves in the surrounding vegetation. Both *Drosophila* larvae and adults harbor a low complexity microbiota (<30 taxa) dominated by fermentative bacteria within the genera *Acetobacter* and *Lactobacillus*, which they acquire from the environment while feeding, similar to mosquitoes, black flies, sand flies, filth flies, and midges (Chandler et al. 2011; Staubach et al. 2013; Wong et al. 2013; Adair et al. 2018).

In addition to its amenability to genetic study, which has made *D. melanogaster* the most well-established invertebrate model of human genetics, the low complexity of the *D. melanogaster* gut microbiota, ease of raising axenic and gnotobiotic fruit flies on defined diets, and recent advances in high-throughput sequencing and genomics techniques have concomitantly made *D. melanogaster* a well-established model of host-microbiota interactions across metazoans broadly. Studies in *D. melanogaster* have collectively helped identify the cellular and molecular basis of roles for gut microbiota in shaping host health and conditioning of host immune defenses, as well as the genetic and environmental factors that influence host-microbiota interactions in different ecological contexts (Chandler et al. 2011; Staubach et al. 2013; Adair et al. 2018). Of noted importance is the role of the gut microbiota in regulating nutrient and/or co-factor provisioning and host signaling during larval development (Shin et al. 2011; Storelli et al. 2011; Ridley et al. 2012; Newell and Douglas 2014; Wong et al. 2014; Douglas 2018; Sommer and Newell 2019; McMullen et al. 2020), as has also been described in mosquitoes and tsetse flies. Gut microbiota may also serve to protect hosts from pathogenic microbes via induction of the fly immune system and/or direct competition between invading pathogens and residential microbes (Lhocine et al. 2008; Broderick et al. 2014; Combe et al. 2014; Lee and Kim 2014; You et al. 2014). For example, studies in *D. melanogaster* indicate that the expression of genes underlying pathogenicity in human pathogens like *Vibrio cholerae* is regulated in part by interactions with commensal microbiota (Fast et al. 2018, 2020). Studies have also been used to demonstrate pathogen inhibition by commensal bacteria commonly used as probiotics in humans (Su et al. 2019), underscoring the value of this species as a model in which to study host responses to microbiota derived from diverse host species.

Finally, *Drosophila* are also commonly infected by intracellular endosymbionts like *Wolbachia* and *Spiroplasma*, and a key element in the use of *Wolbachia* for the control of insect-borne disease has been the discovery that some *Wolbachia* strains can interfere with insect viruses in *Drosophila* and can be readily transinfected into related dipteran insects such as mosquitoes (Xi et al. 2006). For example, mosquitoes transfected with the *w*MelPop-CLA *Wolbachia* strain (derived from *D. melanogaster*) exhibit CI and resistance to nematodes and bacteria (Kambris et al. 2009), viruses such as dengue (Moreira et al. 2009; Bian et al. 2010; Carrington et al. 2018; Flores et al. 2020) and Chikungunya (Moreira et al. 2009), and the avian and rodent malaria parasites *Plasmodium gallinaceum* (Moreira et al. 2009) and *P. berghei* (Kambris et al. 2010). More recent work has also characterized impacts of *Drosophila*-derived *Wolbachia* strains on phenotypes other than pathogen resistance in mosquitoes, including induction of CI and the reduction of host lifespan, blood feeding success, and metabolism (Evans et al. 2009; Kambris et al. 2009; McMeniman et al. 2009; Moreira et al. 2009; Suh et al. 2009; Turley et al. 2009). Interestingly, while natural *Wolbachia* strains that infect mosquitoes have also been shown to induce resistance to viruses (Glaser and Meola 2010), resistance phenotypes in mosquitoes transinfected with *Wolbachia* strains like *w*MelPop-CLA are more robust than in response to natural infections (Moreira et al. 2009). Certain mosquito species, including the main vectors for dengue fever (*Ae. aegypti*) and malaria (*Anopheles* spp.), are also not generally known to be naturally infected by *Wolbachia* but can be transinfected with strains from other host species, including *Drosophila* (Rasgon et al. 2006; McMeniman et al. 2008, 2009; Jin et al. 2009; Bian et al. 2010; Kambris et al. 2010).

The success of *Wolbachia* transinfection for the control of pathogen transmission by dipteran disease vectors fundamentally depends on the ability of *Wolbachia*-infected individuals to spread and invade uninfected populations. That many *Wolbachia* strains both induce CI and confer resistance to pathogens provides an inherent mechanism for *Wolbachia* spread and persistence, given the fitness advantages of infected females over uninfected females. However, any fitness costs (*e.g.*, reduction of host lifespan) associated with infection must be low or absent compared to any fitness advantages. Such fitness impacts are likely to be mediated in large part by interactions between infecting *Wolbachia* strains and the native gut microbiota, which could alter *Wolbachia* densities to the benefit or detriment of associated resistance and/or host fitness. *Wolbachia* infection may concomitantly induce changes in gut microbiota composition and abundance to the benefit or detriment of host health, in the same way 'dysbiosis' of gut microbiota by antibiotics has been shown to be deleterious to several organisms, from plants to humans (Francino 2016; Lee et al. 2021b). Indeed, recent studies have demonstrated both positive (Ye et al. 2017) and negative relationships between *Wolbachia* and Acetobacteraceae abundance in *D. melanogaster* (Simhadri et al. 2017; Moghadam et al. 2018; Rudman et al. 2019). *Wolbachia* densities in *D. melanogaster* also rise in response to antibiotic treatment (Ye et al. 2017). The specific impacts of such *Wolbachia*-gut microbiota interactions on fly fitness have not been explicitly examined, although the well-documented impacts of *Acetobacter* on nutritional phenotypes in *D. melanogaster* and other *Drosophila* spp. strongly suggests that more research is warranted (Shin et al. 2011; Ridley et al. 2012; Newell and Douglas 2014; Sommer and Newell 2019). Future research is also warranted to better understand how variation in host and environmental factors impact gut microbiota diversity, interactions with *Wolbachia,* pathogen resistance and other phenotypes of interest, given that results across different studies are not fully consistent and gut microbial diversity and function is known to vary as a function of host diet as well as both host and microbiota genotypes independent of *Wolbachia* infection (Murdock et al. 2012; Hughes et al. 2014; Dada et al. 2021).

Challenges for harnessing dipteran-associated microbiota for vector and disease control

By altering vector competence and other important biological traits, it is now well established that gut microbiota have the potential to affect the vectorial capacity of dipteran disease vectors to transmit human pathogens. As such, there is a growing interest in developing strategies for manipulating the gut microbiota of mosquitoes and other dipteran insects for disease control. Paratransgenesis is one such approach that leverages genetic manipulation of a target vector's associated microbial community for the production of molecules that inhibit the colonization of potential pathogens or otherwise modulate vector fitness. Such strategies have been explored in the context of malaria vectors in the mosquito genus *Anopheles* (Wang and Jacobs-Lorena 2013). Alternatively, approaches could be used to increase the prevalence and abundance of unmodified gut microbes that naturally inhibit pathogen colonization or vector fitness and that are already present in a target vector population.

Significant challenges must be addressed before implementation of microbe-based control strategies to limit vector-borne disease. Firstly, any taxa of interest exhibiting anti-pathogen or anti-vector properties must readily form a stable association with the vector host. Colonization of the host should also be highly controlled so as to produce only intended phenotypes and avoid any unwanted effects on host physiology, pathogen resistance, and/or vector competence. Lastly, a mechanism must exist to reliably disseminate the microbe into target vector populations in the field. Such methods could include, but are not limited to, the use of sugar bait traps (Mancini et al. 2016; Bilgo et al. 2018) to inoculate adult insects with a sexually or vertically transmitted microbe of interest or the introduction of microbes directly into larval habitats. The latter case poses additional challenges, as the microbe must not only be able to survive within the larval environment long enough to colonize its intended host, but also must be able to persist through the processes of molting and pupation to be transstadially transmitted to newly emerged adults.

In addition to overcoming the above challenges, the long-term success of microbe-based intervention strategies would likely be impacted by factors such as host genetic variation and abiotic gradients that independently modulate vector susceptibility to pathogen colonization (Murdock et al. 2012). Moreover, the natural vector-associated microbiota composition varies spatially, temporally, and relative to laboratory populations. This raises caveats in the interpretation of functional studies in the laboratory that may not accurately represent natural disease dynamics in the field and suggests we should expand the search for candidate microbes in space and time across natural populations. A comprehensive understanding of vector-associated microbiota also involves rectifying the historical bias towards bacteria. Indeed, recent research has elucidated roles for fungi in modulating vector host fitness and in the production of molecules with anti-pathogen activities (Tawidian et al. 2019). Altogether, models of colonization dynamics, persistence, and ecological interactions remain poorly resolved in vector-associated microbial communities but will likely be important in identifying suitable microbial candidates for vector control. Isolating varied and representative microbial communities as well as developing methods for selective colonization of diverse vector species with microbes of interest under different pre-existing colonization and environmental conditions will allow for progress towards assessing microbial candidates of interest across naturally occurring biotic and abiotic gradients.

Diptera as an ideal group in which to study the ecology and evolution of animal-microbe interactions

In addition to serving important roles as bioindicators, biocontrol agents, sources of nutrition for other organisms, and nuisance pests or vectors of disease, which has stimulated broad interest in understanding the mechanisms that have shaped their evolution and diversity, the insect order Diptera is an ideal group in which to study the evolution of animal-microbe interactions because it is monophyletic, highly diverse, and consists of species that are either aquatic during their immature stages or terrestrial, or that exhibit a wide range of habits during the larval and/or adult stages, including predation, parasitism, detritivory, and saprophagy (Fig. 1) (Grimaldi and Engel 2005; Yeates and Wiegmann 2005; Courtney et al. 2009). Many dipteran species are also routinely reared in the laboratory and amenable to genetic studies (Wiegmann and Richards 2018), and methods exist for producing axenic and gnotobiotic individuals, allowing for functional studies (Coon et al. 2014, 2016b, 2020; Koyle et al. 2016). In this way, studies across Diptera have the unique potential to provide novel insights into the relative contributions of life history and phylogeny in shaping gut microbial diversity and function across the animal tree of life. The potential to identify patterns in host-microbiota interactions that are conserved as a function of life history, phylogeny, and/or microbial dependency also has important implications for the development of novel strategies to manipulate interactions to the benefit or detriment of different host species, including dipteran disease vectors and the pathogens they transmit.

Conclusion

The order Diptera contains the most important arthropod vectors, contributing to a significant proportion of infectious disease burden worldwide. Increasingly, research has highlighted the diversity and function of gut-associated microbes across dipterans with direct implications for aspects of their physiology and ecology. A more comprehensive understanding of dipteran-gut microbiota interactions is essential for accurately predicting vectorial capacity and disease transmission dynamics in the field. As a species-rich, monophyletic, phylogenetically resolved, and ecologically diverse group, dipterans are also an ideal model to study the evolution of host-microbe interactions across animals. Future studies should aim to expand experimentally tractable models across the dipteran phylogeny to elucidate functional roles of the gut microbiota with relevance to disease transmission.

Acknowledgements We thank members of the Coon laboratory at University of Wisconsin-Madison (Serena Zhao, Holly Nichols, Sebastián Díaz, and Miguel Medina Muñoz) for fruitful conversations that benefited the writing of this manuscript. Miguel Medina Muñoz also helped with figure preparation.

Author contributions AAA and AJS wrote the initial manuscript, and KLC contributed to revisions.

Funding Writing of this manuscript was supported by awards from the National Science Foundation (2019368), U.S. Department of Agriculture (2018-67012-29991), and the Dairy Innovation Hub at the University of Wisconsin-Madison (to KLC). AAA was further supported by a National Institutes of Health Biotechnology Training Fellowship (NIGMS-5-T32 GM135066), a Howard Hughes Medical Institute Gilliam Fellowship (GT14993), and the SciMed Graduate Research Scholars Program at UW-Madison.

Declarations

Conflict of interest The authors declare that they no conflict of interest.

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